No. 16.—SEPTEMBER, 1904

DEPARTMENT OF THE INTERIOR
BUREAU OF GOVERNMENT LABORATORIES
BIOLOGICAL LABORATORY

PROTECTIVE INOCULATION AGAINST ASIATIC CHOLERA

(AN EXPERIMENTAL STUDY)

BY

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MANILA
BUREAU OF PUBLIC PRINTING
1904

20230

LETTER OF TRANSMITTAL.

DEPARTMENT OF THE INTERIOR,
BUREAU OF GOVERNMENT LABORATORIES,
OFFICE OF THE SUPERINTENDENT OF LABORATORIES,
Manila, P. I., July 6, 1904.

SIR: I have the honor to transmit herewith and recommend for publication a paper entitled "Protective Inoculation Against Asiatic Cholera," an experimental study by Richard P. Strong, M. D., Director of the Biological Laboratory.

I am, very respectfully,

PAUL C. FREER,
Superintendent Government Laboratories.

Hon. DEAN C. WORCESTER,
Secretary of the Interior, Manila, P. I.

PROTECTIVE INOCULATION AGAINST ASIATIC CHOLERA.

(AN EXPERIMENTAL STUDY.)

By Richard P. Strong, M. D., *Director Biological Laboratory.*

The experimental work which forms the basis of this article was for the greater part performed during the spring of 1903 in the Institut für Infektionskrankheiten," Berlin (Prof. R. Koch, director, department of Prof. A. Wassermann). I wish here publicly to express my very grateful thanks to Professor Wassermann, under whose direction the research was first undertaken, for many suggestions and courtesies during the course of my studies. I also wish to express my gratitude to my colleague, Dr. P. C. Freer, for having kindly read the manuscript.

INTRODUCTION.

The epidemic of Asiatic cholera which has recently passed through these Islands has brought forcibly before us the particular difficulties encountered in combating and controlling a disease of this nature in a tropical country and among a partly uneducated people. Moreover, its history has demonstrated that it has not been possible to eradicate or even satisfactorily to control the malady in this city by ordinary hygienic methods—that is, by those measures solely directed toward the purification of the food and water supply of the infected districts. During the period in which the number of infected individuals was the greatest, it was shown by studies made in this Laboratory that in Manila at least the disease was not usually transmitted directly by water,[1] but probably more often by food infection. While cholera is not to be regarded, even in our present acceptation of the term, as a "contagious malady," undoubtedly in this epidemic the infection spread largely though as a rule indirectly, it is true, from

[1] In other portions of the Archipelago the disease was certainly conveyed and spread by the water supply.

case to case. Thus, it was shown how, under the conditions existing here, an individual suffering with cholera or convalescent from it might frequently infect the food of several or many other healthy persons, and thus serve as the true means of continuing and spreading the disease. However, even at that period of the epidemic when general quarantine and isolation of each case discovered was carried out, the malady still continued to increase. While apparently almost every precaution practicable was taken by the Board of Health in regard to the furnishing of uninfected water and the prohibition of the sale of many fruits and other uncooked foods, and while also strenuous efforts were made in the isolation and treatment of the sick and the disinfection of their excreta, although the epidemic was partially held in check, nevertheless, as stated above, it spread, continued for nearly two years, and caused the death of 3,866 people in the city of Manila alone.[1] Hence, it was evident that, at least with a population of this character, it was not possible to prevent many individuals from coming into contact with and even ingesting the cholera organism. It therefore seemed advisable to immunize artificially and to protect by vaccination against the disease as many of this class of people as possible.

However, a few preliminary trials with Haffkine's method of protective inoculation showed the impracticability of using it in these Islands. First, because of the severe local and general reaction which it occasions when a good bactericidal immunity is obtained, the natives would not voluntarily submit to it; and second, on account both of this violent reaction and of the unsettled condition of the country, it was impracticable, or at any rate inadvisable, to make such vaccination compulsory. Lastly, while this inoculation gives rise to a bactericidal and agglutinative serum in the inoculated, the antitoxic value of such serum is probably very slight.

Because of the great importance of this question to the Government of these Islands, an experimental study was undertaken with the object of obtaining some practicable and efficacious form of protective inoculation against the disease. However, before proceeding directly to these studies, it will be appropriate to review briefly the investigations which have hitherto been made in this direction.

[1] In the provinces there were 90,745 deaths from Asiatic cholera reported by Maj. E. C. Carter, Commissioner of Public Health, during the epidemic.

A REVIEW OF THE METHODS OF PROTECTIVE INOCULATION PREVIOUSLY EMPLOYED.

The methods which have been employed for human protective inoculation against cholera are not numerous. Ferran, in 1885 in an epidemic which raged in Spain during that year, was the first to introduce the vaccination of human beings against the disease. He injected guinea pigs with small quantities of bouillon cultures which were inoculated directly from human cholera stools, and found that, in those animals which recovered, a certain immunity had been acquired, since, after a short time they resisted the injection of fatal doses of cholera spirilla. From these observations he decided to experiment upon human beings. His original method of vaccination was apparently for a time kept secret. It was supposed that eight drops of a bouillon culture of the cholera organisms, mixed with bile, were injected subcutaneously, and after an interval of from six to eight days, a second inoculation of 0.5 cubic centimeters of the same mixture was given; eight days later this second dose was repeated.

Subsequently, Ferran stated that his method consisted in using nothing more than a pure culture of the "comma bacillus" in bouillon, of which the dose was 1 cubic centimeter in each arm. Five days later revaccination was performed, the same amount being again injected. The subcutaneous introduction of the living cholera spirilla in this manner did not cause a general infection or give rise as a rule to alarming symptoms, though fever, malaise, lassitude, sometimes diarrhea, and always a considerable local reaction about the point of inoculation became manifest. It is said that about thirty thousand persons were vaccinated, but apparently no reliable statistics were obtained. Several government commissions were appointed to investigate Ferran's method, and their opinions in regard to its merits were usually unfavorable.[1] In general, it may be said that the inoculations, as they were carried on, were considered worthless. According to several of the reports the cultures employed were often not pure ones, nor was there any fixed virulence obtained for the organism used, and even the number of bacteria in a single injection varied greatly, so that an accurate regulation of the dose was not possible. The inoculations

[1] Shakespeare's report spoke more favorably than the others of the results obtained.

finally became so disastrous that they had to be discontinued by the Spanish Government.

In 1888 Gamaleia reported that he was able to immunize guinea pigs and pigeons against fatal doses of the cholera spirillum by the injection of sterilized virulent cultures of this organism. He therefore suggested that this method be employed in human protective inoculation against the disease, emphasizing the advantages of such a chemical vaccine on account of the easy regulation of the dose as well as its sterility. Later he pointed out that after the destruction of the bacilli by heat, they produced only a moderate local reaction upon subcutaneous inoculation.

In spite of the bad results attending Ferran's work in Spain, Haffkine decided, as a result of animal experimentation, that successful active immunization in man could be obtained after Ferran's method, if it were rightly applied.

Haffkine's method of vaccination was as follows: An attenuated virus was first prepared by growing the cholera organism in flasks of bouillon at 39° C. while allowing a constant current of air to pass over the surface of the media. After this attenuation the germs were grown upon agar and carried from tube to tube. A virulent virus was prepared by inoculating a guinea pig intraperitoneally with cholera spirilla and then inoculating a second pig with the peritoneal exudate of the first, and so on through numerous animals until a very virulent culture was obtained. This was known as the fixed virus.

Haffkine maintained that the inoculation of guinea pigs with nonlethal doses of this fixed virus protected them not only against subsequent subcutaneous and intraperitoneal injections of cholera spirilla in lethal doses but also against the introduction of these organisms into the intestine or stomach after neutralization of the gastric juice.

The vaccination of human beings was performed in two stages. In the first 0.1 to 0.05 of a twenty-four hour agar tube of the attenuated culture suspended in bouillon was injected subcutaneously. In the second, performed from three to eight days after the first, the same amount of the virulent culture or "fixed virus" was inoculated. Haffkine states that on the injection of the attenuated culture only a slight local reaction was obtained, consisting merely of edema, and that no necrosis of the tissues took place. This

preliminary vaccination he also says modifies the reaction of the second.

Tamancheff showed that the addition of carbolic acid, in the proportion of 0.5 per cent, killed the organisms without, however, interfering with their immunizing properties. Local reaction and other toxic effects were also diminished, as shown in three human experiments. Haffkine also recommended carbolic acid in a 0.5 per cent solution for sterilizing the cultures after they have been grown upon agar.

Kolle first determined accurately that specific protective substances enter into the serum of human beings inoculated subcutaneously with cholera organisms; for, though Klemperer in 1892 made the same assertion, his experiments were not entirely conclusive. Kolle maintained that a single injection of the living cholera vibrios gave as good an immunity as when the inoculation was repeated, and further that the use of dead cultures produced about the same results as that of living ones. He vaccinated human beings and found that in those who had received a single inoculation of the killed vibrios the serum showed as good an immunity as in those which had been vaccinated several times with living organisms. Patients whose blood serum before inoculation showed a value of 0.75 and 0.6, ten days afterwards showed one of 0.003—that is, 0.003 grams of their serum protected guinea pigs against ten times the fatal dose of the cholera organism. After demonstrating that neither heat nor chloroform destroyed the value of the virus, he recommended the following method for human inoculation:

A well-grown agar culture containing about 20 milligrams of growth was suspended in 10 cubic centimeters of physiological salt solution and sterilized for a few minutes at 50° C.; 0.5 per cent phenol was added to the preparation without apparently interfering with the effectiveness of the virus. In vaccinating, 1 cubic centimeter, equal to 2 milligrams of the culture, was injected subcutaneously. Larger amounts, as high as one-fifth of a culture (4 milligrams), were occasionally employed by Kolle. Haffkine also sometimes used dead cultures, but thought that the living organism gave a greater degree of immunity and a more prolonged one.

The numerous observations made by Haffkine and others in India speak decidedly for the effectiveness of his own as well as of Kolle's vaccine and for the protection which is afforded

by them when properly applied. However, such methods will probably never come into general use, owing to the great discomfort and sometimes even serious results to which they give rise in the inoculated. A few hours after the subcutaneous injection of sufficient amounts of the living or killed virulent cholera spirilla into the human being, the local reaction becomes manifest. There is extreme infiltration in the vicinity of the injection and severe pain on pressure or even on the slightest movement of the inoculated extremity. The temperature rises to 39° or 40° C. There is faintness, general malaise, loss of appetite, and often severe headache and backache. After from one to three days the general and local symptoms usually begin to subside, although the local reaction may persist for a considerably longer time and may even go on to suppuration. Indeed, it may be said that the subcutaneous injection of living or killed virulent cholera vibrios produces even more marked general symptoms and local reaction than the injection of either killed plague or typhoid bacilli. Therefore, it seems probable that, with such a vaccine, the reaction is so great that the method is not likely to be generally submitted to voluntarily.

Moreover, while there is no doubt that the subcutaneous inoculation of living or killed cholera spirilla gives rise to a bactericidal and agglutinative serum, it is very doubtful whether any very great toxic immunity is to be obtained by such injections. It must also be stated that when the bacterial bodies of the cholera organism are injected subcutaneously, in order to obtain a good immunity, a sufficient number to give rise to a severe local reaction must be introduced. The subcutaneous injection of small amounts of avirulent cultures of the cholera organism may be performed with the production of but slight discomfort.

The methods recited above are the only ones which have been extensively employed in the protective inoculation of man against Asiatic cholera, though a few isolated experiments in which other methods were used have been performed on human beings.

Thus, Klemperer in 1892 sought to obtain immunity in man and animals by the subcutaneous injection of the milk of goats which were immunized with the cholera organism. He maintained that the injection of 5 cubic centimeters of the milk of these animals produced such an immunity in man that 0.25 cubic centimeters of the blood serum of the inoculated individual was sufficient to protect guinea pigs against fatal doses of the cholera organism. Ketschner

also claimed similar results by the subcutaneous injection of the milk of goats, which animals had been immunized with cholera spirilla, but when the milk was administered through the mouth, no immunity was obtained. Later Klemperer inoculated himself subcutaneously with 3.6 cubic centimeters of a cholera culture sterilized by heating, and a short time afterwards his blood serum in doses of 0.25 cubic centimeters protected guinea pigs against doses of this organism fatal for the normal animals. Two weeks later he began to ingest killed bouillon cultures of the cholera spirilla, feeding himself one-half liter in divided doses for a period extending over twelve days. He then found that his blood serum in doses of 0.01 protected guinea pigs against lethal amounts of the cholera organism. Hence, it was twenty-five times stronger than before he began the feeding. No unfavorable symptoms from the ingestion of the spirilla were observed.

Sawtschenko and Sabolotny also performed feeding experiments upon themselves and their laboratory assistants. They used agar cultures in which the organisms were killed by successive heatings at 60° C. to 70° C. The bacteria were then suspended in normal saline solution, the liquid evaporated on a water bath and the organisms finally resuspended, sufficient carbolic acid being added to make a 0.5 per cent solution. After showing that their undiluted blood sera did not protect guinea pigs against a fatal dose of the cholera organism, they commenced to ingest the killed carbolized cultures, the experiment extending over a period of four weeks. During this time Sabolotny ingested an amount equal to 1.398 grams and Sawtschenko one equal to 0.838 gram of the dried bacteria. They stated that during their experiment slight symptoms of nervous depression and heaviness in the head were present. At the end of the time mentioned above their sera were collected and injected into guinea pigs in amounts of 1.5, 1.0, 0.5, 0.1, and 0.01 cubic centimeters. After three days the animals were all injected intraperitoneally with a twenty-four hours' agar culture, in amounts equal to 0.0006 of dried bacteria (twice the lethal dose). All these animals recovered, while one control animal without serum injected with 0.0003 gram of the dried cholera organisms died. Guinea pigs injected with 0.005 gram of the serum of Sawtschenko plus 0.006 gram of the dried bacteria also died. Death occurred also when the dose of agar emulsion was increased to 0.003 gram (ten

times the lethal dose), when even 0.5 cubic centimeters of the serum of each individual did not save the guinea pig.

In order to immunize themselves more completely, these authors continued their feeding experiments, Sabolotny finally ingesting 2.318 grams and Sawtschenko 1.758 grams of the dead bacteria. Then, in order to prove themselves immune to cholera infection, a few days after the last dose of the killed organisms, they neutralized their gastric juice with 100 cubic centimeters of a 1 per cent soda solution, and a little later ingested 0.1 cubic centimeter of a twenty-four hours' bouillon culture of the living cholera spirilla. No symptoms followed, although it was stated that cholera organisms were isolated from the stools in each case. This same culture injected into two rabbits in doses of 1 and 0.5 cubic centimeters caused the death of both animals.

While we now recognize that by feeding cultures of bacteria in large amounts a certain degree of immunity may be in some cases gradually developed, yet the method is tedious and very uncertain and has obtained no practical application. Moreover, in the case of toxines, with the exception of ricin, abrin, and certain snake poisons, the results are still more unfavorable for the production of toxic immunity by absorption through the normal gastric or intestinal mucosa.

Various other vaccines against the cholera spirillum, some of them chemically prepared, have also been described.

Brieger and Wassermann in 1892 prepared a virus by growing the cholera spirillum in bouillon prepared with the thymus glands of calves. The organisms were then killed by heating for fifteen minutes at 65° C. or for ten minutes at 80° C. and placed in the ice box for twenty-four hours. By the use of 4 cubic centimeters of this prophylactic in divided doses they were able to protect guinea pigs against three times the fatal dose for normal animals of the cholera vibrio.

Federoff obtained similar results with doses of 1 cubic centimeter of cultures grown in thymus bouillon for from seven to ten days at 37°C., sterilized by heating for fifteen minutes at 65°C., then allowed to stand in a dark room for twenty-four hours and finally mixed with an equal volume of glycerine.

In 1893 Wassermann prepared an extract of the organism in the following manner: One thousand cubic centimenters of a three to five day cholera bouillon culture were evaporated at 70° C. to 80° C.

to a sirupy consistency. The residue was treated with absolute alcohol and the heavy precipitate then filtered off and dried over sulphuric acid. 0.02 gram of this substance, when placed in the peritoneal cavity of a guinea pig, caused the death of the animal; but when 0.005 gram was injected the animal recovered and was found to be immune against ordinarily fatal intraperitoneal doses of the cholera organism.

A different procedure was recommended by Klebs who, after sterilizing cultures of the organism, filtered and concentrated them on a water bath. By precipitation with absolute alcohol the toxic substances were said to be separated out. These were then filtered and the filtrate used for experiments on immunization. Klebs called the preparation "anticholerin" and demonstrated its protective effect upon guinea pigs. He also recommended it for treatment in cases of Asiatic cholera.

Rosmainski in 1894 precipitated sterilized cultures with acetate of lead, removed the lead with oxalic acid, concentrated the filtrate, again precipitated with milk of lime, and finally sterilized the filtrate. This fluid was said to contain protective substances. When the cultures were precipitated with ammonium sulphate the precipitate dried and separation from the ammonium sulphate accomplished, with chloroform, the amorphous powder obtained was found also to possess immunizing properties.

Recently the Swiss Serum and Vaccine Institute of Berne has prepared a prophylactic against cholera obtained from the organism by the method which Lustig and Galeotti described for the preparation of their plague prophylactic. The agar cultures of the organism are dissolved in a 1 per cent caustic potash solution and then treated with 1 per cent acetic acid. The resulting precipitate is filtered off and washed to a neutral reaction and finally dried in a vacuum. Two milligrams of this nucleo-proteid dissolved in 1 cubic centimeter of a soda solution are recommended for the inoculation.

In 1894 Issaeff worked along the line of inducing immunity by increasing the natural resistance of the individual. He found in numerous experiments that different substances, such as tuberculin, nucleic acid, blood serum, bouillon, urine, and even physiologic salt solution, when injected intraperitoneally into animals, caused a transitory protection against infection with cholera spirilla. All of these substances possessed in common the faculty of calling forth

either a local phagocytosis in the peritoneal cavity or a general leucocytosis; the resistance declined as the number of leucocytes returned to normal. In the majority of cases, after four or five days this immunity had already begun to disappear. If, however, microörganisms were injected during this period, specific bactericidal substances entered into the blood and were demonstrable after from three to five months.

Buchner and Hahn sought to obtain immunizing substances from bacterial cells by special mechanical means. These authors triturated masses of the moist cholera bacteria mixed with infusorial earth and fine quartz sand. The organisms were subsequently subjected to a pressure of from four to five hundred atmospheres. The extract thus produced (the so-called cholera plasmin), when injected into guinea pigs in sufficient amounts, gave rise to the same results as the incorporation of the living cholera vibrios. The animals succumbed in from twelve to twenty-four hours, with a marked fall of body temperature. By a single injection of 0.5 cubic centimeters of this cholera plasmin, guinea pigs were protected against ten times the fatal dose of the living cholera organisms. This immunity was found to exist after from three to four months. The animals also showed an agglutinating serum. It may be added that typho-plasmin and tuberculo-plasmin were also prepared, which, it was maintained, also gave good results in the treatment of typhoid fever and tuberculosis.

Emerich and Loew showed that many bacteria, not only in the animal body but also in cultures, secrete enzymes, which in sufficient concentration are able to dissolve the organism producing them. They studied the sediment of old bouillon pyocyaneus cultures and found that most of the organisms were dissolved. In earlier cultures they observed that agglutination preceded this process and could be traced back to the saturation of the bacterial membrane with the enzyme. The pyocyaneus enzyme dissolved not only *Bacillus pyocyaneus,* but also cholera spirilla, anthrax, diphtheria and plague bacilli, as well as *Staphylococcus pyogenes aureus* and *Streptococcus pyogenes.* These authors maintained that natural immunity in man is based upon the presence of bacteriolytic enzymes in the blood, which possibly originate from the bacteria of the intestines. They designated these ferments as "nukleasen" and proposed the terms "pyocyanase" and "cholerase," respectively. For the production of these enzymes, a special culture medium contain-

ing asparagin, peptone, dicalcium phosphate, sodium acetate, chloride of sodium, and magnesium sulphate, was employed. After the growth of the bacteria the liquid medium was neutralized, filtered and evaporated at 25°C. to 30°C. until reaching one-tenth of its original volume, after which it was dialyzed. By the injection of the "pyocyanase" thus obtained animals could be successfully immunized against the injection of various bacteria, among them, the organisms of cholera, anthrax and plague. Rabbits received 12 cubic centimeters intravenously and 7 cubic centimeters subcutaneously without any ill effect and were fully protected.

Other authors, including Dietrich, were not able entirely to confirm this work by experimental investigation, though the damaging effect of "pyocyanase" upon anthrax bacilli, both in vitro and in the animal body, has been confirmed by Tavernari, Krause, and others.

Behring and Ransom, as well as Metchnikoff, Roux, and Taurelli-Salimbeni, showed the possibility of producing a soluble poison from a highly virulent cholera culture. Behring and Ransom obtained a toxine by the filtration of cultures, of which one-fourth cubic centimeter was said to kill guinea pigs of 300 grams' weight in eighteen hours, with all the characteristic appearances of a cholera infection. Metchnikoff and Roux found that by growing the organism in collodion sacs in the abdominal cavity of guinea pigs and afterwards upon a special medium containing gelatin mixed with serum a soluble toxine had been produced, which killed guinea pigs when administered subcutaneously in amounts of one-third cubic centimeter per 100 grams' weight. Experiments in the immunization of animals, especially of horses, were made. After six months' treatment a horse furnished a serum, 1 cubic centimeter of which had the power of neutralizing four times the fatal dose of the cholera poison. It was maintained that this serum was protective for animals not only against the cholera toxine but also against the injection of living vibrios and even against infection by way of the stomach. Practical use of these toxines for protective inoculation in man has not as yet been made.

Passive immunization against Asiatic cholera by the use of antitoxic or bactericidal sera need not be discussed here, as it is obvious that from the standpoint alone of the brief immunity which they confer, the use of either for a practical prophylactic would not be satisfactory.

Finally Besredka performed experiments upon animals and a single inoculation upon himself with cultures treated in the following manner: The organisms were grown upon agar for twenty-four hours and suspended in normal saline solution, after which enough immune serum was added to cause complete agglutination. The mixture was allowed to stand for twelve hours, during which time the amboceptors and uniceptors of the serum became fixed to the bacteria. The clear fluid was then poured off and the agglutinated organisms freed from the excess of serum containing unbound amboceptors by repeated washings with normal saline solution. The residue, consisting of organisms plus the immune bodies, was heated to 56° C. for one hour and then injected subcutaneously. In the experiment which Besrekda performed upon himself the plague bacillus was the organism employed, but similar results were obtained in animals in which the cholera spirillum was treated by the same method. He maintained that in this manner immediate immunity was conferred, which lasted for at least five to six months, and that no local or general symptoms followed the injection. He attributed the lack of a general reaction in his own case to the non-toxicity of the vaccine, and the absence of a local reaction he thought was probably due to the fact that, when the immune body was fixed to the bacilli, the latter became the prey of the phagocytes almost instantly.

He stated that the early appearance of the immunity may be explained in two ways, both according to the theory of Metchnikoff. He first refers to the idea that the immune body carried by the bacteria becomes free in the animal organism and thus acts as a preventive serum, that is to say, favors phagocytosis of the organisms introduced. Thus the immunity of the animal is assured in the beginning. He is, however, inclined to discard this explanation, since in the case of plague it required a period of forty-eight hours before the immune body manifested its preventive action. He therefore believed the immune body to have no other function than that of increasing and stimulating the work of the phagocytes in such a manner as to enable them to accomplish their action in a shorter time than would be possible in its absence. In this manner the time necessary to give rise to active immunity would be notably shortened.

It is obvious that another theoretical explanation can be given for the results of Besredka's experiments. Thus, it is clear that

in his prophylactic at the time of injection the haptophore groups of the bacteria were not all saturated with amboceptors and hence were capable of exerting certain immunizing power. Evidently, however, such power must be reduced below that possessed by the same bacteria (without serum) with no bound haptophore groups. The amboceptors which were introduced united to the bacteria could of course theoretically, after the destruction of the microörganisms in the body, exert a passive immunizing effect. However, the number of amboceptors injected in this manner would be less than that introduced in a moderate dose of immune serum alone.

From this brief review, therefore, we are apparently justified in drawing the conclusion that as yet no satisfactory form of human protective inoculation against cholera, effective as well as practical, has been established, since those prophylactics, which give rise to the necessary immunity produce too severe a local reaction, while no other, causing only a mild local reaction, has been shown to be equally or sufficiently protective. However, of the methods which have been employed, those of Haffkine and Kolle promise the best results.

I shall now refer to my experimental work upon this subject.

DESCRIPTION OF THE CULTURES EMPLOYED.

In my search for a practical vaccine, I first studied the local reaction and other toxic effects produced in animals after the injection both of a very virulent cholera culture and of one which, through cultivation on artificial media for a long period of time, had lost most of its virulence. The effects of the killed as well as of the living organisms were also studied with each culture. These two stems, for the sake of brevity, will be referred to in this article as "virulent" and "avirulent."

The avirulent organism was obtained through the kindness of Professor Wassermann. It had been isolated by R. Pfeiffer in the epidemic of cholera which occurred in Hamburg in 1894, and for nine years was preserved on artificial media in the laboratory and from time to time passed through animals. During the past year, however, the strain employed in these experiments had only been grown on artificial media and not inoculated into animals. Its growth on all culture media was typical for *Spirillum choleræ asiaticæ*, including the production of indol in proper peptone solution,

demonstrable by the addition of nitrite-free sulphuric acid. In its morphological characteristics it was not typical, in that short commas were seldom observed, but instead rather long, thread-like forms predominated; also its motility was to a great extent, though not entirely, lost. However, it was agglutinated by a standard cholera immune serum in even higher dilutions than is another genuine cholera strain; and there is no doubt whatever of its representing a true though attenuated type of *Spirillum choleræ asiaticæ*.

The virulent organism was isolated by Professor Kolle in Jaffa during the recent epidemic of cholera in that place. It reacted in all media in a perfectly typical way, and its morphology and motility were also characteristic of the genuine cholera organism. It is agglutinated in high dilutions by the same cholera immune serum, though not in so great ones as the avirulent strain.

Some time was spent in accurately standardizing these cultures, and the minimal lethal dose for guinea pigs of 250 grams' weight was carefully determined. After numerous passages of "virulent" through animals, a lethal dose of 0.1 of a standard (2 mg.) *oese*[1] of a twenty-hour agar culture was reached. Such a dose of "virulent," when suspended in 1 cubic centimeter of an 0.85 per cent sodium chloride solution and injected intraperitoneally into a guinea pig of 250 grams' weight, regularly caused death within twenty-four hours. With "avirulent," on the other hand, one and one-half standard *oesen* of a twenty-hour agar culture, when injected intraperitoneally, were required to produce death within the same time in such an animal. The former strain, therefore, may the said to possess fifteen times the virulence of the latter. Throughout the course of the work this relationship between the organisms has been carefully preserved and continually tested by animal inoculation. As the virulence of cholera spirilla grown on laboratory media changes in a few days, it is necessary to make daily animal inoculations in the case of the virulent strain and always to use the same generation of the stem. With the avirulent culture considerable care was also necessary to keep its virulence fixed.

INOCULATIONS WITH LIVING CHOLERA ORGANISMS.

It soon became evident that the local reaction upon the tissues after the subcutaneous injection of "avirulent" was much less

[1] This standard *oese* was employed throughout the work.

TABLE No. I

The average weight of rabbits, 1500 grams.
The injections were made into an ear vein with ½ oese of the living organisms suspended in 1 c. c. bouillon. All animals were killed by bleeding one week after inoculation. Agglutination experiments performed with both stems, virulent and avirulent; Bactericidal reactions performed only with the virulent stem.

Rabbit No.	Inoculated with ½ Oese intraven.	Agglutination Experiments. Organism	Dilution of serum. 1:50	1:100	1:200	1:300	1:400	1:500	1:600	1:700	1:800	Controls NaCl sol.	Controls no serum	Bactericidal reactions (Pfeiffer's Phenomenon.) Dilution of serum 1:50	1:100	1:200	1:300	1:400	1:500	1:600	1:700	1:800	1:900	1:1000	1:2000	1:4000	1:6000	1:8000	1:10000	1:12000	1:14000	1:15000	Control Animals Without Serum.
V.	"Virulent"	"virulent"					W	N	N	N	N	N	N																		N	6 control animals all neg; all dead in 24 hours.
		"avirulent"								N	N	N	N	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	D	
VI.	"Virulent"	"virulent"					W	W	N	N	N	N	N																		N	9 control animals all neg; all dead in 24 hours.
		"avirulent"									W	N	N	A	A			A						A	A	A	A	A	A	A	D	
VII.	"Virulent"	Died after five days. Agar plate cultures from all organs were sterile																														
VIII.	"Avirulent"	"virulent"				W	W	N	N	N	N	N	N																N	N		5 control animals all neg; all dead in 24 hours.
		"avirulent"						W	W	N	N	N	N	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	D			
IX.	"Avirulent"	"virulent"			W	W	N	N	N	N	N	N	N																N			8 control animals all neg; all dead in 24 hours.
		"avirulent"							W	N	N	N	N		A			A						A	A	A	A	A	D			
X.	"Avirulent"	"virulent"			W	N	N	N	N	N	N	N	N																N			7 control animals all neg; all dead in 24 hours.
		"avirulent"							W	N	N	N	N	A	A			A						A	A	A	A	A	D			

W = Complete agglutination. Overlying liquid clear.

W = Distinct agglutination with precipitation. Overlying liquid not entirely clear.

A = Microscopically, positive bactericidal reaction. Animal alive after 24 hours.

N/D = Microscopically, negative bactericidal reaction. Animal dead after 24 hours.

+/A = Microscopically, Pfeiffer's Phenomenon doubtful; Animal alive after 24 hours.

than after one with the "virulent" cholera spirillum. I therefore decided to determine the character of the serum which could be produced with this avirulent organism and to compare it with that produced by the virulent germ. Accordingly a series of six rabbits of an average weight of 1,500 grams was inoculated intravenously, three each with one-half *oese* of "virulent" and three each with one-half *oese* of "avirulent," the organisms in every instance being suspended in 1 cubic centimeter of bouillon. After eight days the rabbits were all killed by bleeding and the value of the serum in each case determined for agglutinative and bactericidal properties. It then became evident that the rabbits inoculated with the virulent culture always furnished better serum than those inoculated with the avirulent one, but that the value, in both agglutinative and bactericidal properties, of the serum from the animals treated with the former was in no case more than two and one-half times that of the serum furnished by the animals treated with the latter stem. The results of the experiments may be seen in detail in Table I.

TECHNIQUE EMPLOYED.

The technique of the agglutinative and bactericidal reactions employed throughout the work was as follows:

The reactions for agglutination were performed in the test tube. One *oese* of the living organism was thoroughly suspended in 1 cubic centimeter of an 0.85 per cent solution of sodium chloride. The amount of serum to be tested, suspended in 1 cubic centimeter of a similar saline solution was then added, the tube well shaken, and the mixture allowed to stand two hours at 37° C. In a complete agglutination it is understood that the liquid overlying the precipitated bacteria appears entirely clear. By a weak reaction we understand one in which there is a distinct agglutination with precipitation of numbers of the organisms, visible to the naked eye, but in which the supernatant fluid remains more or less cloudy.

The bactericidal reactions were performed in the abdominal cavity of guinea pigs according to the well-known method of R. Pfeiffer, a hypodermic syringe with a blunt-pointed needle being employed for the injections, care being taken to avoid any injury to the intestine during the inoculation. The dilutions of the serum were made in normal saline solutions. One cubic centimeter of the diluted serum was then added to 1 cubic centimeter of bouillon

containing 2 *oesen* of "virulent" in suspension, after which 1 cubic centimeter of the resulting mixture was injected into the peritoneal cavity of a guinea pig of 250 grams' weight (or a little less), the animal thus receiving ten times the fatal dose of the living organisms. A fresh guinea pig was of course used for each reaction. The experiment was controlled by microscopic examination of a drop of serum from the abdominal cavity, made immediately and again twenty minutes after the inoculation, and obtained by means of a capillary tube, and by the inoculation of control animals with ten times the fatal dose of "virulent" but without serum. The result to the animal after twenty-four hours, whether it was then living or dead, was regarded as the final test, though the condition of the organisms in the abdominal cavity after twenty minutes was always carefully noted.

CONTINUATION OF THE EXPERIMENTS WITH THE INOCULATION OF THE LIVING ORGANISMS.

As some outcome such as was obtained in the experiments given in Table I was not entirely unexpected, but as the results were somewhat at variance with the ideas of Haffkine and quite different from what R. Pfeiffer and Friedberger found upon the intravenous injection into rabbits of dead cholera spirilla of different degrees of virulence, it was decided to repeat them. Accordingly, a second series of animals was inoculated just as the first, and on the day of inoculation, as in the previous series, the virulence of the injected organisms was verified as fifteen to one. The result was practically the same, for at the end of eight days the examination of the sera showed that the virulent stem had in only one case given a serum of more than about two and one-fourth times the bactericidal value of that produced by the avirulent one. In this one case the avirulent serum was between one-fourth and one-fifth as strong. (See Table II.)

We shall not discuss here in detail the interpretation of these results or attempt to explain the discrepancy in immunity in comparison with the relation of virulence between the two stems. This will be done in another paper. It will be sufficient perhaps to state here that apparently from the results of these experiments with the intravenous injection of living organisms into rabbits in amounts of one-half *oese* we might assume that the immunity produced is not directly proportional to the virulence of the inoculated organisms.

TABLE No. II.

Inoculations with living organisms
average weight of rabbits 1150 grains.
(For explanation see Table No. I.)

Rabbit No	Inoculated with ½ Oese intraven.	Agglutination Experiments. Organism	Dilution of Serum. 1:50 1:100 1:200 1:300 1:400 1:500 1:600 1:700 1:800 1:900 1:1000	Controls No. II: Salt: 1% serum	Bactericidal reactions (Pfeiffers Phenomenon) Dilution of Serum 1:100 1:1000 1:2000 1:3000 1:4000 1:5000 1:6000 1:8000 1:9000 1:10000 1:15000 1:16000 1:17000 1:18000 1:19000	Control Animals Without Serum.
194	Virulent	"virulent"	A A A	N N	A A A A A A A A A D	2 control animals, reactions neg.; all dead in 24 hours.
		avirulent	W N N	N N		
195	Virulent	"virulent"	W	N N	A A A A A ± N D	1 control animal, reaction neg.; dead in 24 hours.
		avirulent	W	N N		
196	Virulent	"virulent"	N N	N N	A A A ± N A D	2 control animals; reactions neg. all dead in 24 hours.
		avirulent	W N	N N		
197	Avirulent	"virulent"	N N	N N	N N A A A A A A A A D	2 control animals, reactions neg: all dead in 24 hours.
		avirulent	N N	N N		
198	Avirulent	"virulent"	N N	N N	± A A A A A A A D	2 control animals, reactions neg: all dead in 24 hours.
		avirulent	W N	N N		
199	Avirulent	"virulent"	N	N N	N A A A A A D	1 control animal; reaction neg.; dead in 24 hours.
		"avirulent"	N	N N		

In the experiments performed with the subcutaneous injection of the avirulent organism, in both the living and the dead state, while the local reaction, as already mentioned, was much milder than with the use of the virulent germ, there was always considerable inflammation about the point of inoculation, and it soon became evident that it would be highly desirable, if possible, to do away entirely with the bacterial cells.

In this connection it was necessary to recall that probably a satisfactory protective against Asiatic cholera must contain substances which give rise to antitoxic as well as bactericidal bodies in the blood sera of the inoculated. Hence, a consideration of both these substances was necessary. Accordingly, the problem of their extraction from the bodies of the bacteria was next investigated.

It may be said by way of parenthesis that in this connection, on reviewing clinical experience with Asiatic cholera, I thought of the conditions under which the toxines are set free in the human body in cases of this disease. In epidemic cholera it is very evident that the symptoms usually develop very rapidly after they have once begun, and, indeed, usually within a few hours after their appearance, either death or a severe state of collapse has supervened. The cholera process can not be satisfactorily explained except as the effect of an organic poison, and it therefore appears not unlikely that in a very short space of time a considerable amount of toxic material is manufactured and liberated. Further, if cultures are prepared from the intestinal material made during the period of the most acute symptoms and compared with those taken forty-eight hours or a longer period after the entrance of the stage of collapse, it will be found that in the latter condition there is usually a remarkable reduction in the number of cholera spirilla and apparently a great increase in the other intestinal bacteria. If this clinical experience is compared with the observations made in the laboratory, namely, that in agar cultures of cholera spirilla the maximum growth at 37° C. is obtained in from twelve to twenty hours and that after this time a rapid death of the spirilla takes place, so that according to Gotschlich and Weigang, after two days only 10 per cent (at the maximum) and after three days only 1 per cent (at the maximum) of the organisms which were present at the end of the first twenty-four hours are still alive; it may be supposed that perhaps the stage of the most violent symptoms in human cholera may correspond with the period during which the rapid

dying off of the spirilla occurs. This would then correspond to the facts observed in the laboratory cultures, and it might be presumed that it is at the time of death of the spirilla that the largest amount of toxine is set free.

It may be difficult to establish the correctness of such an hypothesis; but when one considers the work which has been performed in the laboratory in regard to the chemistry of the cholera vibrio, the facts are certainly suggestive of such a possibility. Since, moreover, this subject has a direct bearing upon the extraction of the immunizing properties of the organism, it will here be appropriate briefly to refer to these experiments and to examine more closely into the chemistry of this spirillum.

THE CHOLERA TOXINE.

Since the publication of the first article of R. Koch, in which it was held that the cholera paroxysm, and especially the algid state, was to be regarded as a specific intoxication, due to the absorption from the intestine of the metabolic products of the cholera organism, many authors have attempted to produce experimental proof of the existence and to isolate this specific toxine. Hueppe was one of the first to investigate this question. He and his pupils believed the toxine to be formed in the intestinal tract under nearly anærobic conditions, and attempted to reproduce experimentally the relations which exist in the human intestine. Accordingly, fresh hen's eggs were inoculated with the cholera vibrio. This organism was said to give rise to hydrogen sulphide produced from the proteid material after using up the oxygen present, thus bringing about anærobic conditions. With such relations Hueppe maintained that the specific toxine was formed in large amounts.

However, it has been shown subsequently by several authors (among them Doenitz and Zenthoffer) that the cholera organism in sterile egg culture media neither produces sulphuretted hydrogen nor, indeed, multiplies to any great extent under anærobic conditions; so that one is forced to the conclusion that Hueppe's media was not free from other microörganisms. The toxine which he obtained was probably one resulting from putrefaction due to anærobic bacteria. Scholl and Gruber, from cultures on eggs, also obtained a similar toxic peptone, which the latter was able to precipitate with alcohol. The work of Nicati and Rietsch, Pouchet, Villiers, Cantani, Kunz, Brieger and Fraenkel, Petri, Winter and

LeSage, Klebs, Gamaleia, Gruber and Sluyt, need only be referred to here. To-day it would appear that none of these authors were dealing with the specific cholera toxine alone.

R. Pfeiffer studied the filtration of old and young bouillon cultures of the cholera organism. He found that the filtrate of cultures from one to five days old possessed no poisonous action after injection into animals. Only old cultures which had grown for many weeks or months contained a soluble poison, which, upon filtration, caused the death of the animals inoculated. These poisonous materials, however, behaved in a manner entirely similar to that of the basic alkaloidal bodies, to which the name of ptomaines has been given. Their action became manifest in a very brief period of time and in a manner entirely different from that of the cholera poison. Further investigation, in which immunization was attempted with such products, made it certain that these dissolved substances which were contained in old cholera cultures had nothing to do with the primary and secondary cholera toxines. They are basic bodies which may be found in many old cultures of bacteria and are without any specific significance. However, R. Pfeiffer was able to show that in fresh agar cultures of cholera bacteria the bacterial cells contained a powerful toxic substance. If such cultures were carefully killed through short contact with chloroform vapor, or by heating at 65° C. for one hour and were then injected intraperitoneally in small quantities into guinea pigs, death resulted, even though such cultures were sterile. Ten milligrams of an eighteen-hour culture, which was exposed for ten minutes to the action of chloroform vapor, caused the death of a guinea pig of 200 grams' weight with all the symptoms of a true cholera intoxication. This intracellular poison showed considerable instability. According to Pfeiffer and Wassermann, through treatment with various chemicals—absolute alcohol, concentrated solutions of neutral salts, etc.—or by boiling or prolonged heating at 60° C., a change from the primary to the secondary cholera poison takes place. Therefore, in cultures which are killed at high temperatures, a less toxic effect may be expected than in those killed at lower ones, as the latter contain only the primary poison. The action of the primary toxine, from a physiological standpoint also, is different from that of the secondary.

The earlier work of Pfeiffer was performed with the organism known as "cholera Massowah," which is now known not to be a

genuine cholera spirillum. However, his work has been confirmed for the latter organism by numerous authors, among them Kolle and Wassermann, and at present there is apparently but little doubt that the true cholera toxine exists as a constituent element of the bacterial cell. However, up to the present time we have not been successful in obtaining it in a pure state. This poison apparently becomes soluble only through the disintegration of the bodies of the bacteria. In all fluid cultures the growth of the vibrios is after a time prevented through plasmolysis, plasmotysis, or digestion, and the bacteria die. Thus, the soluble toxine is set free, but in these cultures it seems to be very unstable and is soon destroyed.

On the other hand, other observers, principally Metchnikoff, Roux, and Taurelli-Salimbeni, all working together, show that the living cholera germ produces a soluble diffusible toxine. These authors base their claims upon the following experiments:

A small, sterilized collodion sac, of a capacity of three or four cubic centimeters, was half filled with peptone solution or nutrient bouillon, inoculated with the cholera spirillum, closed and placed in the abdominal cavity of a guinea pig. Another guinea pig received a similar sac containing an emulsion of one and one-half gelatin cultures of cholera bacteria suspended in peptone solution and killed by chloroform, while a sac containing only peptone solution was placed in a third animal. The last animal remained unaffected. The one which received the emulsion of dead bacteria showed a slight elevation of temperature and emaciation, while the animals which had received the living organisms died in from three to five days with all the appearances of cholera intoxication. The collodion sac in these animals still showed motile spirilla.

In order to produce this soluble toxine in artificial media, a highly virulent organism was grown in similar sacs in the abdominal cavity of a guinea pig. In this way a culture was obtained, one-one hundred and sixtieth cubic centimeter of which sufficed to kill guinea pigs. This organism was then grown in a culture medium consisting of 2 per cent gelatine, 2 per cent peptone, and 1 per cent sodium chloride with the addition of fresh guinea pig serum from another sac. Cultures from this medium after three or four days, when filtered, killed guinea pigs in from sixteen to twenty-four hours, on being administered in amounts of one-third cubic centimeter per 100 grams of body weight. The toxine thus obtained

was not materially changed on being boiled, but lost its toxicity on contact with the air and on exposure to light. With such a toxine it was maintained that a highly effective antitoxic serum could be produced in animals. Attempts made by some other competent observers to repeat these experiments have not as yet been successful.

It seems that there are objections to some of the conclusions of Metchnikoff and Roux. In the first place results of experiments made with collodion sacs it would appear are not entirely confirmatory of their ideas. As has already been intimated, the organisms confined in a collodion sac, in the abdominal cavity of an animal, would after twenty-four hours die in large numbers, and through plasmolysis and disintegration the toxine would be set free from the bacterial cells. The living organisms remaining would later give rise to additional toxine in the same way, and thus the death of the animal would eventually result. In the case in which the dead organisms were inoculated in the sacs, there was, as stated by these authors, an elevation of the temperature for several days and emaciation. The amount of cholera toxine present was obviously not sufficient to bring about the death of the animal.

To-day no one would suppose that the same virulence is to be expected from the dead organisms as from the living ones, and particularly would this be true in a closed collodion sac. With the living organisms there would be many successive generations from which additional amounts of toxine would be furnished. It is stated that at the conclusion of the experiments made by these authors living vibrios were still present. Hence, the total amount of toxine set free would be many times larger than that from one generation of the killed germ. Moreover, it is not improbable that with organisms killed by chloroform and placed in collodion sacs, the intracellular toxine is not likely to be entirely given up, unless some further disintegration of the cells takes place. In other words, such conditions are not favorable to digestion and plasmolysis, a point which will be referred to in a later paper.

It is difficult to confront such evidence as Buchner brings forth in his work on cholera plasmin, in which the toxine was extracted by grinding and pressing the bacteria. The following experiments, which may easily be performed, also seem very difficult to explain on any other assumption than that the toxine exists in the body of the organism:

Two, eighteen-hour agar cultures are taken and the growth of each suspended in sterile normal saline solution. No. 1 is filtered through a Reichel candle and the filtrate is injected into guinea pigs in varying amounts. It is then seen that this filtrate possesses very little toxic power. On the other hand, if what remains on the filter be injected, even though the organisms are killed before injection, the animal dies with all the symptons of cholera intoxication. Evidently the bacteria contain the toxine.

If the second culture is carefully killed by heating to 60° C. for a brief period and the bacteria are allowed to digest themselves by their own ferments for two or three days, ground, pressed in a hydraulic press, and then filtered off, the filtrate obtained from these killed and digested organisms, when injected into animals, shows toxic properties. Regarding the so-called toxines of Behring and Ramson, it seems likely, as Pfeiffer has remarked, that the cholera toxine which they have written of does not represent the primary poison, but rather secondary toxines and alkaloids which originate in old cultures and from which, indeed, Brieger has obtained cadaverin. In still older cultures he found putrescin, cholin, methylguanidin, and other toxic substances.

In connection with the subject of toxine production it may be appropriate to refer to the investigations of Kraus. This author performed his work with an organism designated as *Vibrio Naskin.* It is certain that this organism was not a genuine *Spirillum choleræ asiaticæ,* for in a cholera immune serum which agglutinated several strains of the cholera spirillum in dilutions of 1:20,000 *Vibrio Naskin* was not agglutinated in dilutions higher than 1:400, and furthermore, the serum obtained with *Vibrio Naskin* agglutinated it in dilutions of 1:800, but did not affect the cholera organism. *Vibrio Naskin* also produced a hemolysine which was not neutralized by a cholera-immune serum, but was by an anti-Vibrio Naskin serum. Moreover, the precipitines of the two organisms were not identical. From this vibrio (Naskin) Kraus was able to obtain a powerful toxic substance, which caused death in rabbits, guinea pigs, and other animals. The filtrates of the bouillon cultures were also toxic. By heating to 50° C. the poisonous properties were destroyed. This toxine could not be identified with the one obtained from the cholera spirillum by Metchnikoff. However, it is interesting to note the production of a soluble toxine in a vibrio of this nature and to call attention

to the caution that must be exercised in the recognition of a cholera spirillum to be used in experiments relating to toxine formation.

From this review it is apparent that as yet no substantial evidence has been brought forth to show that the cholera spirillum produces, as do the diphtheria and the tetanus bacilli, a powerful soluble toxine. Even in fresh cholera bouillon and peptone cultures (two or three days old) there are found numbers of dead bacteria, from which, through plasmolysis and digestion, the intracellular toxines are set free. The most reliable evidence points to the conclusion that this toxine exists as an integral part of the bacterial cell, and I believe that in the remarks which are to follow I shall be able to give further proof of this point.

THE FERMENTS OF THE ORGANISM.

The increased attention given to the importance of ferments in physiological and pathological processes, for example, in the self-digestion of tissues and cells which often results from the activity of certain intracellular ferments, led me to investigate the action of the enzymes of the cholera spirillum upon its own protoplasm, and the effects of the digestive products which are formed. The cholera vibrio, under certain conditions, produces at least four of these enzymes.

Bitter first demonstrated the diastatic action of this organism and found that it develops an acid in nutrient solutions containing starch paste. Fermi succeeded in obtaining this ferment in a pure condition, not only in the case of the cholera spirillum but also with several other varieties of microörganisms. He demonstrated that it is formed in culture media free from starch, but that, on the contrary, in those free from proteid it is not produced. A temperature of 60° C. destroys or markedly decreases the activity of the ferment.

The inverting ferments, as is known, are not frequently produced by bacteria; but Fermi and Montesano succeeded in demonstrating that the cholera vibrio, in either sugar or proteid-free media, sometimes, though very inconstantly, produces ferments of this class.

The cholera spirillum has been shown by Schoffer to produce a rennet-like ferment, which is similar in its action to the rennin of the cow's stomach. Fokker showed that the action of this ferment was destroyed by a temperature above 60° C.

It is, however, with the production of its peptonizing ferment that we are most concerned at present. Bitter first showed that

the liquefying of the gelatin in cultures of this organism is due to a real ferment action and that it occurs independently of the living bacterial cell. A cholera culture in which the organisms had been killed by heating to 60° C. still showed intense peptonizing action. This author was able to obtain this ferment in a pure condition by the following method:

Sixty-five per cent alcohol was added to gelatin which had been liquefied by the spirillum. In this manner the proteid, but not the ferment, was precipitated. After twenty-four hours the precipitate was removed by filtration and the ferment was precipitated from the filtrate by the addition of absolute alcohol. It was found that, when collected on a filter and dried, the ferment could be dissolved in an aqueous solution of thymol and its peptonizing properties demonstrated on gelatin tubes. Like similar ferments, it converts an indefinite amount of coagulated albumen into peptone, and it is more active in alkaline than in acid solutions; thus resembling trypsin more than pepsin. A small amount of acid prevents its action.

A noticeable property of these peptonizing ferments in general is their great resistance to dry heat. Thus, for example, the ferment of *Vibrio Finkler-Prior* is said to resist heating for ten minutes at from 120° C. to 140° C. However, they are less resistant when subjected to moist heat, the same ferment then becoming inactive at 70° C. Damaging influences, such as light or poisons, which either kill the bacteria or prevent their growth, also affect the action of these enzymes, though sometimes they are more resistant toward certain chemical substances than the bacteria or even the spores of the latter. These ferments can digest and sometimes peptonize not only gelatin but also coagulated serum, egg albumen, fibrin, and casein of milk.

We need only mention the work of Sommaruga in regard to the production of a fat-splitting ferment by the cholera spirillum, since there seems to be some doubt as to whether such a ferment is actually produced by this organism. It is apparently not formed in ordinary media.[1]

[1] C. Oppenheimer (Die Fermente und ihre Wirkungen, 1903, p. 290) states that Carriére found a lipase in cholera bacilli, but it would appear that the tubercle bacillus was the organism from which Carriére isolated this ferment. (G. Carriére: Sur L'Existence d'un Ferment Soluble dans les Cultures de Bacilles de Koch, Comptes Rendus Societe de Biologie, vol. 53, p. 320, 1901.)

It may also be added here that Kraus and Clairmont have recently again called attention to the phenomenon first noticed by Koch, namely, the hemolytic action of cholera cultures.

The question arose then as to what might be the action of this peptonizing ferment of the cholera spirillum upon cultures of this organism. Here it is necessary once more to refer to the work of Gotschlich and Weigang, who, as mentioned above, found that in agar cultures after forty-eight hours only 7.43 per cent, and after sixty-eight hours only 0.8 per cent of the cholera spirilla which were present at the end of the first twenty-four hours in the same medium, were still alive. In one of their experiments with an agar culture kept at 37° C. for from fifteen to twenty hours, 10,000,000 individual organisms died; at the temperature of the ice box this rapid death of the bacteria did not result. Indeed, they noted that agar cultures which had been grown at 37° C. for twelve hours and then placed at the temperature of the ice box for twelve hours longer showed a greater number of organisms than did those which were kept continuously for twenty hours at 37° C. Conradi investigated this question further and concluded that this rapid destruction of the organisms resulted from the action of certain degenerative products formed autolytically within the cultures of the bacteria. He demonstrated that, if cultures in which further growth had been arrested, were placed in reed sacs, impervious to bacteria and the autolytic bactericidal substances then removed by dialysis, a new growth could be observed. He also explained Gotschlich and Weigang's results, which showed that the death of organisms kept at the temperature of the ice box is less rapid, by the demonstration that at this temperature the enzymes of the organism are not capable of exerting any marked chemical action.

PREPARATION OF THE PROPHYLACTIC.

After a consideration of the data given above and acting upon the supposition (1) that the cholera toxine exists as an integral part of the bacterial cell, and (2) that it is set free after the death of the organism and probably partly through the action of its own proteolytic enzyme, which is not destroyed at 60° C., I determined to find out whether the other immunizing substances (agglutinine and bacteriolysine) as well as the toxine could not be separated from the bodies of the bacteria by a process of autolytic digestion.

Accordingly cholera spirilla from stems known to possess good

peptonizing powers were placed in an aqueous solution, carefully killed by heating and digested at 37° C. It was then found that the cholera receptors were set free in the fluid in great abundance, a fact which after filtration was easily demonstrable, since such filtrates possessed the power of binding uni- and ambo-ceptors (agglutinines and bacteriolysines) in a cholera-immune serum, as well as the ability, after injection into rabbits, of giving rise to the appearance of toxic symptoms, and in the case of the ultimate recovery of the animal, to the entrance of antitoxic, bactericidal, and agglutinative substances in the blood serum. Therefore, a filtrate prepared in this manner immediately recommended itself for trial as a prophylactic.

It was prepared in large quantities after the following manner: The surfaces of large flat-sided flasks (after the pattern of Kolle), filled with agar, were sprayed with twenty-hour bouillon cultures of the organism, and the flasks were then put aside for twenty hours in the incubator at 37° C.[1] After this period the growth was suspended in sterile water, removed from the surface of the agar, and the suspension then placed in a sterile flask at 60° C. for from one to twenty-four hours. The mixture was afterwards put aside in the incubator at 37° C. for from two to five days, and finally filtered through a Reichel candle.

In certain experiments the milky fluid overlying the sediment of the bacteria at the bottom of the flask was poured off and the latter crushed and subjected to hydraulic pressure of 600 atmospheres. Later the extracts from these pressed organisms and the original aqueous solution, previously decanted, were together under pressure passed through a Reichel or Berkefeld filter.

EXPERIMENTS WITH THE PROPHYLACTIC.

INTRAVENOUS INOCULATION.

Since the intravenous inoculation of four rabbits with from 2 to 3 cubic centimeters of a fluid obtained in this manner from the

[1] Cultures of twenty hours' duration were always used, on account of the rapid death of the organisms in older cultures. Fresh beef was employed in the preparation of the media. The agar at the time of use had an alkalinity of 1 per cent to phenolphthaleïn. This gave a sufficient alkalinity to the aqueous suspensions of the organisms to bring about a favorable action of the proteolytic ferment.

TABLE No. III.

Inoculations with "Prophylactic"
(For explanation see Table No. I.)

Rabbit No.	Inoculated with	Agglutination Experiments. Organism	Dilution of Serum. 1-50	1-100	1-200	1-300	1-400	1-500	1-600	1-700	Controls No. Cl sol., no serum.		Bactericidal reactions (Pfeiffer's Phenomenon). Dilution of Serum. 1-50	1-100	1-200	1-300	1-400	1-500	1-600	1-700	1-800	1-900	1-1000	1-1500	1-2000	1-3000	1-4000	1-5000	1-7500	1-10000	1-15000	Control Animals Without Serum.
58	"Virulent"	"virulent"					N	N	N	N	N	N																				5 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I	"avirulent"					W	W	N	N	N	N	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	D			
59	"Virulent"	"virulent"				W	N	N	N	N	N	N																				4 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I	"avirulent"					W	W	N	N	N	N	A	A	A	A		A		A			A	A	A	D						
60	"Virulent"	"virulent"						N	N	N	N	N																				3 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I	"avirulent"						W	W	N	N	N	A	A	A	A		A		A			A	A	A	A	D					
61	Avirulent	"virulent"					W	N	N	N	N	N																				4 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I	"avirulent"						N	N	N	N	N	A	A		A		A		A	A	A	D	D								
62	Avirulent	"virulent"			N	N	N	N	N	N	N	N																				3 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I	"avirulent"			W	W	N	N	N		N	N	A	A		A		A	A	A	A	A	D									
63	Avirulent	"virulent"				N	N	N	N		N	N																				3 control animals; reactions neg.; all dead in 24 hours.
	Prophylactic I	"avirulent"				N	N	N	N		N	N	A	A		A		A	A	A	A	A	D									

virulent strain, in which, however, the organisms were heated for only one hour at 60° C., caused the death of all the animals and showed the extract to be powerfully toxic, it was thought advisable to attempt to weaken this toxic action by a more prolonged heating of the organism, in order that the bactericidal and agglutinative immunity might be studied in the inoculated animals. Accordingly, for the next series of rabbits, twenty-hour agar cultures of the organism were suspended in sterile water, placed at 60° C. for twenty-four hours, digested for two days at 37° C., and finally passed through a Reichel filter. For the sake of comparison, both stems, the virulent and the avirulent, were treated in this way, and the filtrates were labeled respectively "Virulent Prophylactic I" and "Avirulent Prophylactic I." One cubic centimeter of each filtrate represented the number of receptors obtained from 2 *oesen* of the living organisms. The rabbits were of about the same average weight (1,500 grams) as those used in the experiments of Table I. Each animal was inoculated intravenously with 1 cubic centimeter of the filtrate (equal to 2 *oesen*). After eight days they were all killed by bleeding and the bactericidal and agglutinative values of their blood sera were carefully determined. The results may be seen in Table III.

Thus we see from animals Nos. 58 and 60 that, with the intravenous injection into rabbits of 1 cubic centimeter of virulent prophylactic I, there were obtained sera showing an agglutinative value with the virulent stem of about 3.3 and 2.5 milligrams, and with the avirulent one of about 2.0 and 1.66 milligrams, and a bactericidal value against the former strain of 0.09 and 0.08 milligram. However, in the rabbits inoculated with the avirulent prophylactic the sera were not of nearly so great a value, showing an agglutinative worth of only about 10.0 to 3.3 milligrams, and a bactericidal one of 1.1 to 0.5 milligrams. Indeed, on comparing the sera of animals Nos. 58 and 60 with those of animals Nos. 62 and 63, we see that those of the two former possess about thirteen and fourteen times as great a bactericidal value as those of the two latter (0.09 and 0.08 milligram against 1.1 milligrams.) However, in the case of animals Nos. 59 and 61 this proportion was not maintained, the bactericidal value of animal No. 59 representing only three times that of animal No. 61.

This series of experiments suggested that by the use of this method of autolytic digestion a more favorable result, that is a bet-

ter bactericidal immunity, was to be obtained with the virulent organism, and that the immunity acquired was within certain limits directly dependent upon the virulence of the stem used in the preparation of the virus. The value of the sera obtained from the animals inoculated with the virulent prophylactic also offered encouragement for a more extensive trial of this method with certain modifications.

Experiments with prophylatic II.—Therefore another quantity of the prophylactic was prepared, some slight changes being introduced in the method. Twenty-hour agar cultures of the virulent organism were suspended in sterile distilled water and the suspension was then divided into three portions, each being placed in a separate sterile flask and kept at 60° C. for twenty-four hours. The first portion was allowed to digest for two days, and the second and third for five. All three were then filtered separately, after which the third was reheated at 60° C. for two hours. It is necessary to state that with the same amount of organisms, twice as much distilled water was used in preparing the suspension of the agar cultures in the case of prophylactic II as was employed in that of prophylactic I. Hence, 1 cubic centimeter of the former filtrate contained only the number of receptors obtained from 1 *oese* of the living organisms, so that it possessed only one-half the strength of prophylactic I.

Four rabbits were injected intravenously, each with 12 cubic centimeters of this prophylactic, animals Nos. 86 and 87 receiving the portions digested for only two days, animal No. 88 that digested for five days, and animal No. 89 that reheated for two hours at 60° C. after five days' digestion; each animal receiving the number of receptors obtained from the digestion of 12 *oesen* of the living organisms. After eight days the animals were, as usual, killed by bleeding and the values of their sera carefully estimated, as may be seen from Table IV.

The results obtained in this series of experiments gave still greater encouragement for the method and suggested that by a digestion of five days, more receptors were set free from the bacterial cells in an aqueous solution than by one of two days.[1] Animals Nos. 86 and 87, each receiving inoculations of the portions digested

[1] Further experimentation has shown that the best results are obtained with from three to five days' digestion. No better sera were produced with filtrates which had been subjected to digestion for a longer period.

TABLE No. IV.

Inoculations with Prophylactic II.

(For explanation see Table No I.)

Rabbit

No.	Inoculated with 12 C.C. intraven. (1 C.C. = 1 oese)	Organism	Agglutination Experiments. Dilution of Serum. 1-50	1-100	1-200	1-400	1-600	1-700	1-800	1-900	Controls No. CL. Sol. no serum	Bactericidal Reactions (Pfeiffer's Phenomenon) Dilution of Serum 1-100	1000	5000	13000	15000	17000	18000	19000	20000	22000	23000	Control Animals Without Serum
86	Virulent Prophylactic II. digested 2 days	virulent						W	N	N	N N	A	A	A	A	A	A	A	N				2 control animals, reactions neg; all dead in 24 hours.
		avirulent							W	N	N N								D				
87	Virulent Prophylactic II. digested 2 days	virulent					W	N	N		N N	A	A		A	A	A	A	N				1 control animal; reaction neg; dead in 24 hours.
		avirulent							N	N	N N								D				
88	Virulent Prophylactic II. digested 5 days	virulent						W	N		N N		A	A	A	A	A			A	A	N	2 control animals, reactions neg; all dead in 24 hours.
		avirulent								W	N N											D	
89	Virulent Prophylactic II digested 5 days & reheated 2 hrs. 60° C	virulent					W	N	N	N	N N		A	A	A	A				A	D	N	1 control animal; reaction neg; dead in 24 hours.
		avirulent					W	N	N	N	N N											D	

TABLE No. V.

Inoculations with "Prophylactic III."
Digested for five days.
(For explanation see Table No. I.)

Rabbit No	Inoculated with	Agglutination Experiments: Organism	Agglutination Experiments: Dilution of Serum 1:50 1:100 1:200 1:300 1:400 1:500 1:600 1:700 1:800 1:900 1:1000 1:2000	Controls Sort Salt no serum	Bactericidal Reactions (Pfeiffer's Phenomenon): Dilution of Serum 1:100 1:500 1:1000 2,000 4,000 5,000 6,000 10,000 15,000 20,000 25,000 30,000 35,000	Control Animals Without Serum:
192	"Virulent" Prophylactic III	"virulent"	W N	N N	A A A A A A A + A D	6 control animals, reactions neg: all dead in 24 hours.
		"avirulent"	W W A N	N N		
193	"Virulent" Prophylactic III.	"virulent"	W N	N N	A A A A A + A D	5 control animals, reactions neg: all dead in 24 hours
		"avirulent"	W N	N N		
204	"Avirulent" Prophylactic III.	"virulent"	W A N N A	N N	N A A A A D	5 control animals: reactions neg: all dead in 24 hours.
		"avirulent"	W N N	N N		
205	"Avirulent" Prophylactic III.	"virulent"	W N N A	N N	N A A A D	4 control animals: reactions neg: all dead in 24 hours.
		"avirulent"	35 W N	N N		

TABLE No. VI.

Inoculations with "Prophylactic IV." digested for 2 days.
(For explanation see Table No. I.)

Rabbit No.	Inoculated with 12 c.c. intravenously = 1 dose.	Agglutination Experiments. Organisms	Dilution of Serum. 1-100	1-200	1-300	1-400	1-500	1-700	1-800	1-900	Controls Sol. no serum	Controls No. Cl.	Bactericidal Reactions (Pfeiffer's Phenomenon) Dilution of Serum. 1-500	1000	1200	1400	5000	10000	12000	13000	15000	16000	Control Animals Without Serum.
256	"Virulent" Prophylactic IV.	"virulent"						W	N	N	N	N									±	N	1 control animal; reaction neg. dead in 24 hours.
		"avirulent"							N	N	N	N		A			A	A			A	D	
257	"Virulent" Prophylactic IV.	"virulent"						N	N	N	N	N							±	N			1 control animal, reaction neg. dead in 24 hours.
		"avirulent"						N	N	N	N	N		A			A	A	A	D			
177	"Avirulent" Prophylactic IV.	"virulent"			N	N					N	N			N								1 control animal, reaction negative. dead within twenty four hours.
		"avirulent"				N					N	N	A	A	A	D							
178	"Avirulent" Prophylactic IV.	"virulent"		W	N	N					N	N			N								
		"avirulent"			W	N					N	N	A	A	D								

for two days, showed an agglutinative value of about 1.25 and 1.43 milligrams, and a bactericidal immunity of 0.05 milligram; while animal No. 88, inoculated with the portion digested for five days, showed an agglutinative value of 1.1 milligrams, and a bactericidal value of about 0.04 milligram. Experiments with animal No. 89 demonstrated that reheating at 60° C. for two hours had destroyed to some extent the agglutinative substances of the bacteria which was made apparent by an evident loss of agglutinine in the serum, the latter showing a value of only 1.6 milligrams. The substances giving rise to the bactericidal qualities were apparently but little affected by the second heating, since the bactericidal value of the serum of animal No. 89 was between 0.04 and 0.05 milligram, which was only a little poorer than that of animal No. 88 (0.04 milligram).

Experiments with prophylactics III and IV.—Further information being desired as to whether, with five days' digestion, more receptors are always set free in the fluid than after the action of this process for two days, two more portions of the prophylactic were prepared with both strains, one being digested for five and the other for two days at 37° C. In prophylactic III sufficient sterile water was used for the suspension of the organisms so that 1 cubic centimeter of it contained the number of receptors obtained from 2 *oesen* of the living organisms; while in prophylactic IV, 1 cubic centimeter of the suspension equaled the receptors from 1 *oese*. Hence, in the inoculation of the rabbits in which prophylactic IV was employed, double the amount of fluid was injected. A glance at Tables V and VI will explain these experiments. The rabbits which received prophylactic III, which had been digested for five days, all furnished better sera than those injected with the virus of corresponding virulence of prophylactic IV digested for only two days. The best serum obtained from the injection of virulent prophylactic IV was that of animal No. 256, which showed an agglutinative value of about 1.4 milligrams and a bactericidal one of 0.06 milligram; while the best obtained with prophylactic III was from animal No. 192, this one showing an agglutinative limit of 1 milligram and a bactericidal value of 0.04 milligrams. Comparing the experiments of animals Nos. 192 and 193 of Table V with those made with animals Nos. 88 and 89 of Table IV, it will be seen that the rabbits employed in the former case furnished a

slightly better serum. The only difference apparent in the virus, however, was that prophylactic II was not so concentrated as virulent prophylactic III. In other words, even though, as far as could be determined, corresponding amounts of the digested organisms in solution were injected, the animals receiving the more concentrated virus furnished a slightly better serum. However, the differences in value of the sera are so slight that unless constant they might be explained by natural variation in the animals used.

From the experiments of Tables III to VI, it seemed that sufficient data had been obtained in regard to securing a good agglutinative and bactericidal serum by means of a protective prepared after this method, and that its success for intravenous injections was assured.

SUBCUTANEOUS INOCULATION.

The next step, then, was to discover what degree of immunity could be obtained when the prophylactic was injected subcutaneously. Accordingly, with this end in view, experiments were made with both strains, the inoculations being performed subdermally in rabbits. Such a series of experiments is recorded in Table VII, from which it may be seen that after the subcutaneous injection of 5 cubic centimeters of the most favorable virus, sera were obtained with an agglutinative value of from 2.5 to 1.6 milligrams and a bactericidal value of from 0.14 to 0.1 milligram. These results were regarded as very favorable.

EXPERIMENTS WITH THE DRIED PROPHYLACTIC.

In several instances the prophylactic was evaporated in a vacuum at 38° C. and then pulverized, after which it was redissolved in normal saline solution, and injected into rabbits both intravenously and subcutaneously in varying amounts. While a plain loss in the potency of the prophylactic thus treated is evident from the experiments recorded in Table VIII, nevertheless a very good agglutinative serum and bactericidal immunity were obtained. Animal Nos. 167, inoculated intravenously with 10 milligrams of the powder obtained from virulent prophylactic II, produced, after eight days, a serum which agglutinated in dilutions of 10 milligrams, and showed a bactericidal reaction in dilutions of 0.25 milligram. Animal No. 187, inoculated subcutaneously with 5

TABLE No. VIII.

Inoculations with Dried Prophylactic redissolved in No. Ct sol.

(For explanation see Table No I)

Rabbit No.	Inoculated with: Dried Prophylactic intraven.	Agglutination Experiments: Organism.	Dilution of Serum 1-20 1-40 1-50 1-60 1-80 1-100 1-200	Controls No.1 Sol. no Serum	Bacteriodal Reactions (Pfeiffer's Phenomenon) Dilution of Serum 1-50 1-100 1-200 1-300 1-400 1-500 1-600 1-700 1-800 1-1000 1-2000 1-3000 1-4000 1-8000	Control Animals Without Serum
167	10 Mgs. Virulent II	virulent	N	N N	A A A A A A A A A A D	2 control animals; reactions neg.; all dead in 24 hours.
		avirulent	N	N N		
168	3 Mgs. Virulent II.	virulent	N N	N N	A A A A D	2 control animals; reactions neg.; all dead in 24 hours.
		avirulent	W N	N N		
187	(Subcutaneous) 5 Mgs. Virulent III	virulent	N N	N N	A A A A A D	2 control animals; reactions neg.; all dead in 24 hours.
		avirulent	W N	N N		
169	10 Mgs. Avirulent II.	virulent	N N	N N	A A A A A A A D	2 control animals; reactions neg.; all dead in 24 hours.
		avirulent	W N	N N		
170	3 Mgs Avirulent II.	virulent	N	N N	A A A A D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	N	N N		
175	(Subcutaneous) 5 Mgs Avirulent III.	virulent	N N	N N	A A A A A D	1 control animal; reaction neg.; dead in 24 hours.
		avirulent	W N N	N N		

TABLE No. VIII.

Inoculations with Dried Prophylactic redissolved in Na Cl sol.

(For explanation see Table No I)

Rabbit

No.	Inoculated with Dried Prophylactic intraven.	Agglutination Experiments. Organism	Dilution of Serum 1:20	1:40	1:50	1:60	1:80	1:100	1:200	Controls Na Cl sol.	Controls no Serum	Bactericidal Reactions (Pfeiffer's Phenomenon) Dilution of Serum 1:50	1:100	1:200	1:300	1:400	1:500	1:600	1:700	1:800	1:1000	1:2000	1:3000	1:4000	1:5000	Control Animals Without Serum
167	10 Mgs. Virulent II.	virulent							N	N	N														N	2 control animals; reactions neg.; all dead in 24 hours.
		avirulent				I			N	N	N	A	A	A		A	A			A	A	A	A	A	D	
168	3 Mgs. Virulent II	virulent				N	N			N	N						N									2 control animals; reactions neg.; all dead in 24 hours.
		avirulent				W	N			N	N	A	A	A	A	D										
187	(Subcutaneous) 5 Mgs. Virulent III.	virulent						N	N	N	N							N								2 control animals; reactions neg.; all dead in 24 hours.
		avirulent						W	N	N	N	A	A	A	A	A	D									
169	10 Mgs. Avirulent II.	virulent						N	N	N	N										N					2 control animals; reactions neg.; all dead in 24 hours.
		avirulent						W	N	N	N	A	A	A	A		A		A	A	D					
170	3 Mgs Avirulent II.	virulent			N					N	N				N											1 control animal; reaction neg., dead in 24 hours.
		avirulent			N					N	N	A	A	A	I	D										
175	(Subcutaneous) 5 Mgs Avirulent III.	virulent					N	N		N	N						N									1 control animal; reaction neg.; dead in 24 hours.
		avirulent		W			N	N		N	N	A	A	A	A	A	D									

milligrams of the powder obtained from virulent prophylactic III, showed after one week a serum of a weak agglutinative value of 10 milligrams and a bactericidal one of 1.6 milligrams.

STUDY OF LOCAL AND GENERAL REACTION FOLLOWING INOCULATION OF THE PROPHYLACTIC.

Speaking in a general way in regard to the inoculations recorded in Tables III to VIII, it may be said that the animals apparently suffered very little from the injection of the prophylactic, even when very large amounts were employed. Usually they showed a rise of temperature of 1 or rarely 2 degrees during the thirty-six hours immediately following the inoculation. A number of rabbits were treated subcutaneously with each separate lot of the prophylactic, for the sole purpose of observing the local reaction. Only a portion of these were killed subsequently and the value of their sera determined. (See Table VII.) In those animals receiving the injection subcutaneously, the skin was first shaved and subsequently carefully examined in the vicinity of the point of inoculation for any local reaction that might have appeared. Even when large amounts of the virulent prophylactic (5 cubic centimeters) were used, no suppuration ever occurred, and, indeed, induration was very rarely observed. Usually after twenty-four hours there was no trace of a local reaction visible to the naked eye, and upon palpation no induration was evident. These animals also showed a slight and transitory rise of temperature of 1 or 2 degrees.

In regard to the retention of the immunity, it may be stated that several of the animals were killed from three to six months after the inoculation and that at this time an examination of their blood sera still demonstrated a high agglutinative and bactericidal reaction.

COMPARISON OF THE IMMUNITY PRODUCED BY THE VIRULENT AND THE AVIRULENT PROPHYLACTIC.

In comparing the immunity obtained by the use of the virulent and the avirulent prophylactic, we see that on the whole the results recorded in Table III (already referred to) are borne out. It will be recalled that in this table the ratio of bactericidal immunity between the animals treated with the virulent prophylactic and those treated with the avirulent one varied between about $3\frac{1}{2}$ to 1 and 12 to 1. In Table V the sera obtained from the animals inoculated with the virulent prophylactic showed a bactericidal value from

about five and one-half to twelve times as great as that obtained from the injection of corresponding amounts of the avirulent. In Table VI the animals of the "virulent" series showed sera from six to fifteen times as great as those of the "avirulent" ones. In Table VII, with subcutaneous inoculation (Nos. 399, 400, 423, and 184), the proportion is from eight to eleven times as great; and in Table VIII, with the dry prophylactic, the value is from one and one-third to four times as great. The results obtained with the dry prophylactic are certainly not so accurate as those with the fluid, on account of the manipulations to which the powder was subjected; and since they are not in accord with all the other numerous experiments, in which the liquid prophylactic was employed, they must be discarded in this comparative consideration. With this exception the results here reported (with the free receptors) are in harmony with those which have been obtained by other observers who for inoculation have employed strains of the killed organisms of different virulence; namely, that the immunity obtained is within certain limits proportional to the virulence of the inoculated strain.

Upon comparing the immunity obtained by the intravenous injection of the prophylactic into rabbits with that produced in the same manner by the inoculation of the living organisms, we see that by the injection of 1 cubic centimeter of the virulent prophylactic (representing the number of receptors obtained from 2 *oesen* after two days' digestion), there is occasionally obtained a serum nearly equaling in bactericidal and agglutinative properties that produced by the intravenous injection of one-half *ocse* of the living virulent organisms. (Compare the animals comprising Table I with those of Table III, particularly animals Nos. 58 and 60.) By a single intravenous injection of 6 cubic centimeters of the prophylactic (obtained from 12 *oesen* after five days' digestion), a serum of far greater value was produced, namely, one agglutinating in dilutions of 1 to 900 to 1 to 1000 (1 milligram) and showing a bactericidal value as high as 1 to 24,000 (0.04 milligram). (See Table V.) Therefore by a judicious use of this method of autolytic digestion a means is offered us of producing by a single intravenous injection into rabbits a serum of greater bactericidal and agglutinative value than could be produced through the employment in the same manner of either the killed or the living organisms. It is known that an agglutinative value of 1 milligram and a bactericidal one of

0.04 milligram are not to be usually obtained by the single injection of cultures of either the killed or the living cholera vibrios, or even when the inoculation is repeated.

EXPERIMENTS ON GUINEA PIGS, SHOWING THE PROTECTION AFFORDED BY THE PROPHYLACTIC, ETC.

From six to twelve guinea pigs were inoculated intraperitoneally and subcutaneously with varying amounts of each of prophylactics I, II, III, and IV and of dried prophylactics II and III, redissolved in normal saline solution. The dose varied from 1 to 5 cubic centimeters. From seven to ten days after the injection of the prophylactic, the animals received intraperitoneally either five or ten times the fatal dose of the living virulent cholera strain. When the virulent prophylactic was employed the animals were invariably protected; but when the avirulent one was used in small amounts of 1 to 2 cubic centimeters, the guinea pigs sometimes succumbed to the subsequent injection of the living organisms. A few of the animals which received large amounts (5 cubic centimeters), of the virulent prophylactic intraperitoneally succumbed, evidently on account of its toxic effects. Upon autopsy no injection of the vessels or hemorrhages at the point of inoculation were observed, such as are always found when death occurs from the inoculation of the living or the dead organisms. Neither were there any hemorrhages in the serous surfaces of the peritoneum. The most noticeable lesions in these cases consisted of a marked edema of the abdominal walls with some flakes of fibrin over the liver. Microscopically it was observed that an extensive desquamation of the epithelial cells had occurred. The contents of the abdominal cavity were sterile.

However, $\frac{1}{2}$ cubic centimeter of the thick material, consisting of the debris of the bacteria which accumulates at the bottom of the flask in the manufacture of the prophylactic, and remains behind on the filter in the form of an emulsion after the prophylactic is passed through, when injected intraperitoneally, causes the death of guinea pigs, in which are found post-mortem the most extensive reaction locally at the point of inoculation and throughout the abdominal cavity, consisting of hemorrhagic areas, injection of the larger vessels, and corrosion of the subcutaneous tissues. These effects evidently are caused by toxic substances forming a constituent of the bacterial membrane not soluble in aqueous solution

after autolytic digestion and which have probably little to do with the production of the true immunity against the disease.

The following experiment shows the comparative value of the protection furnished by the virulent prophylactic and that given by the injection of the living organisms. Four large guinea pigs of about the same weight were chosen. Two were inoculated intraperitoneally with one-fifteenth *oese* of the living virulent organisms and two with 5 cubic centimeters of the virulent prophylactic IV. One of those which received the living organisms was very sick for twenty-four hours following the inoculation. After six days all the animals were reinoculated intraperitoneally with 2 *oesen* of the living virulent strain. The two which had received the prophylactic previously lived: the other two died. Evidently the protection furnished the guinea pigs by the prophylactic was greater than that furnished by the previous injection of one-fifteenth *oese* of the living organisms, a supposition also borne out, and in a more striking way as previously noted, by comparing the immunity obtained by the intravenous injection of the prophylactic into rabbits with that obtained by the injection of the living organisms. From the foregoing it is evident that the virulent prophylactic forms a reliable and *certain* means of protecting guinea pigs against the subsequent injection of multiple fatal doses of the cholera spirillum.

A more extensive study and trial of the bacterial extracts, consisting of the free receptors of the organism in the preparation of prophylactics and sera, would seem to be advantageous. Certain bacterial extracts already have been shown to be of considerable value. Thus, R. Koch, as early as 1891, prepared his original tuberculin by killing, through heating in the steam sterilizer for one hour, four weeks' glycerin bouillon cultures of the tubercle bacillus, evaporating the cultures to one-tenth of their volume, and finally separating the soluble substances from the bacterial cells by filtration. Behring and Landmann, by a somewhat similar method, prepared extracts from the tubercle bacillus, which were said to possess powerful toxic properties. These authors recommended their extracts for the treatment of early cases of tuberculosis, and for the preparation from horses of a curative serum for the disease. Nocard applied a method, somewhat similar to that employed by R. Koch in the preparation of tuberculin, to *Bacillus mallei* for the production of mallein. Still more recently, Conradi, and Neisser

TABLE No. IX.

Showing toxic action of Prophylactic upon rabbits and effect of carbolic acid and chloretone (used as a preservative) upon this toxine.

Rabbit No.	Inoculated with.	Agglutination Experiments with virulent organism only. Dilution of Serum. 1-100 1-200 1-300 1-400 1-500 1-600		Controls No. of Sol. no serum		Bactericidal Reactions. (Pfeiffer's Phenomenon.) Dilution of Serum. 1-100 1,000 5,000 7,000 8,000 10,000 12,000	Control Animals Without Serum.
395	5 c.c. Virulent Prophylactic V. 1 c.c. = 8 oese.	Dead after 4 hours.					
396	4 c.c. Virulent Prophylactic V.	Dead after 4 hours.					
397	1 c.c Virulent Prophylactic V.		Died after 3 days no serum for further reactions.	N	N		
398	2 c.c Virulent Prophylactic V.	Dead within 24 hours.					
420	1 c.c. Virulent Prophylactic V. Preserved .5% carbolic acid.	N N	Killed after 8 days.	N	N	A A A A A N/D	1 control animal; reaction neg. dead in 24 hours.
421	1 c.c. Virulent Prophylactic V. Preserved .5% carbolic acid.	N N N	Killed after 8 days	N	N	A A A N/D	1 control animal; reaction neg, dead in 24 hours.
422	2 c.c. Virulent Prophylactic V. Preserved .5% carbolic acid.		* " "	N	N	A A A A *	1 control animal; reaction neg; dead in 24 hours
434	3 c.c Virulent Prophylactic V. Preserved .5% carbolic acid.	Dead within 24 hours.					
424	1 c.c Virulent Prophylactic V. reheated to 60°C for 15 min.	Dead within 24 hours					
425	3 c.c. Virulent Prophylactic V. reheated to 60°C for 15 min.	Dead within 24 hours.					
438	1 c.c. Virulent Prophylactic VI. (in chloretone) 1 c.c. = 8 oese.	Dead within 24 hours.					
439	1/2 c.c. Virulent Prophylactic VI. (in chloretone) 1 c.c. = 8 oese.	Dead within 24 hours.					

* No reactions with higher dilutions.

and Shiga,[1] have suggested the use of these extracts in the preparation of a curative serum for typhoid fever and dysentery. However, Conradi advocates the autolytic digestion of the organisms under more natural conditions—i. e., without previous destruction of the bacilli by heat.

We have already begun in the Biological Laboratory experiments relating to the production of a more satisfactory plague prophylactic along somewhat similar lines to those we have employed in the preparation of the cholera prophylactic.

STUDY OF THE TOXIC ACTION OF THE PROPHYLACTIC.

As stated previously, in a few preliminary experiments made with the intravenous injection into rabbits of the virulent prophylactic, if the organisms had been killed by only a very brief period of heating and then allowed to digest themselves, the animals all succumbed to the inoculation. In order to study carefully the agglutinative and bactericidal value of the sera of the inoculated animals, an attempt was made to weaken the toxic action by prolonged heating at 60° C. This heating apparently had the desired effect, as the rabbits then usually survived the inoculations. It then became important to study this toxic action more closely. Accordingly, a prophylactic was prepared by killing the organisms within a very brief period, digesting at 37° C., grinding, submitting them to a pressure of about 600 atmospheres, and finally filtering under pressure through a Reichel or Berkefeld candle. Varying quantities of the prophylactic were then injected intravenously into rabbits. A series of such experiments may be seen in Table IX, and in the case of the control animals of Tables X, XI, and XII.

From these experiments we see that 2 cubic centimeters of virulent prophylactic V prepared after this manner, when injected into rabbits, caused the death of these animals within twenty-four hours. Four and five cubic centimeters injected in the same way produced death in a much shorter time. However, the animals sometimes recovered from the injection of 1 cubic centimeter and were afterwards immune. With virulent prophylactic VI, in which the filtration was performed under pressure with a coarser Berkefeld filter and the bacteria subjected to a more thorough crushing

[1]Since the above was written Shiga has advocated the use of the free receptors as a prophylactic against typhoid fever, and Martin Mayer has obtained interesting results from autolysis of bacteria following precipitation with weak ammonium sulphate solutions.

process, even one-half cubic centimeter caused the death of rabbits. In the animals which succumbed to the inoculation, it was found upon post-mortem examination that the kidneys were swollen and showed other evidences of parenchymatous nephritis. The mesenteric vessels were deeply injected and the liver congested and swollen. The lungs showed patches of congestion, hemorrhages, and in one or two cases small pneumonic areas. In a few of the animals there were hemorrhages in the peritoneal surface of the small intestine.

Heating the organism at 60° C. evidently destroys most of the primary poison, or, at any rate, converts the toxine into toxoid, since it is necessary, in order to bring about the death of the guinea pigs, to inject intraperitoneally relatively large amounts (3 to 5 cubic centimeters) of the heated prophylactic. It would seem that the presence of a toxoid would be more desirable in a human prophylactic than that of the unchanged toxine, the toxoid, through the presence of its haptophore group (although its toxophore group is mainly destroyed) still being able to produce antitoxine in the inoculated body without unfolding its general poisonous effect. Such a result we are able to obtain from the injection of our cholera prophylactic, as may be seen from the experiments recorded in Tables X and XI, which are self-explanatory. From Table X it is evident that 3 cubic centimeters and even 2 cubic centimeters of the serum of a rabbit (animal No. 422), which had previously been inoculated with 2 cubic centimeters of the virulent heated prophylactic V, protected other rabbits against two or three times the intravenous dose fatal for these animals; while 2 cubic centimeters of human serum, obtained from a man previously inoculated subcutaneously with 3 cubic centimeters of virulent prophylactic V, when inoculated into rabbits were capable of neutralizing about four times the dose of toxine fatal for these animals. In the experiments shown in Table XI, it may be seen that one-fifth cubic centimeter of the serum of animal No. 423 (previously inoculated subcutaneously with 5 cubic centimeters of virulent prophylactic V) protected a rabbit against about four times the fatal dose of the toxine. In all of these experiments the prophylactic and the serum were mixed immediately before inoculation. The control rabbits without serum died; this being true also in cases in which equal amounts of normal serum were added to the prophylactic before injection. The results recorded in Tables X and XI were the best

TABLE No. X.—*Showing the antitoxic properties of animal and human serum one week after inoculation of host with the prophylatic.*

Rabbit No.	Inoculated intravenously with—	Result to animal.
432	2 c. c. virulent prophylactic VI (1 c. c. = 8 oesen); no serum	Death within 24 hours.
433	2 c. c. virulent prophylactic VI preserved in 0.5 per cent carbolic acid, plus 2 c. c. serum, animal No. 422.[1]	Apparently unaffected by inoculation; alive after one week.
434	3 c. c. virulent prophylactic V, preserved in 0.5 per cent carbolic acid.	Death within 24 hours.
435	3 c. c. virulent prophylactic V, plus 3 c. c. serum, animal No. 422.[1]	Apparently unaffected by the inoculation; alive after one week.
436	2 c. c. virulent prophylactic VI, plus 2 c. c. human serum, No. 1[2]	Do.
437	2 c. c. virulent prophylactic VI, digested with chloretone (1 c. c. = 8 oesen).	Death within 24 hours.
438	1 c. c. virulent prophylactic VI, digested with chloretone (1 c. c. = 8 oesen).	Do.

[1] Animal No. 422 was inoculated with 2 c. c. virulent prophylactic V intravenously and killed by bleeding one week after inoculation. (See Table IX.)
[2] Human inoculation No. 1 was made with 3 c. c. virulent prophylactic V subcutaneously. Blood was drawn after one week. (See Table XIV.)

TABLE No. XI.—*Showing the antitoxic properties of serum of rabbit one week after subcutaneous inoculation with prophylactic.*

Rabbit No.	Inoculated intravenously with—	Result to animal.
452	2 c. c. virulent prophylactic VII (chloretone) (1 c. c. = 8 oesen)	Death within 3 hours.
454	2 c. c. virulent prophylactic VII (chloretone), plus 1 c. c. serum, animal No. 423.[1]	Apparently unaffected by the inoculation; alive after one week.
455	2 c. c. virulent prophylactic VII, plus one-fifth c. c. serum, animal No. 423.[1]	Do.
456	2 c. c. virulent prophylactic VII, plus 2 c. c. NaCl solution.	Death within 24 hours.

[1] Animal No. 423 was inoculated subcutaneously with 5 c. c. virulent prophylactic V, preserved in 0.5 per cent carbolic acid, and killed by bleeding one week after inoculation. (See Table VII.)

that could be obtained. When smaller quantities of the serum were employed, the death of the animals always resulted. Indeed, neither would 2 cubic centimeters of the immune serum protect against any higher doses of the toxine. It is admitted that such antitoxic values of the sera, obtained from animals previously inoculated with the prophylactic, are not high; though perhaps better antitoxic properties can be produced when more improved methods are employed to extract the intracellular toxine.

In certain experiments recently performed, in which the digested bacteria before filtration were ground in a mortar with fine quartz sand and infusorial earth, a greater toxic effect on the guinea pigs was observed, these animals dying from intraperitoneal injections of one-half to 1 cubic centimeter of such a fluid. Judging from my own experience in this respect it would appear that the most advantageous method for the extraction of the intracellular toxine of the cholera spirillum would be the one which Macfadyen has recently applied with the same end in view to the typhoid bacillus. By this method the bacteria are ground at the temperature of liquid air, the disintegration having occurred under conditions which precluded the possibility of chemical change. It would seem that a combination of these two methods would perhaps furnish a more ideal prophylactic against Asiatic cholera, namely, the method of autolytic digestion which I have described for obtaining the substances which give rise to the bacterial immunity, and the method of Macfadyen for the extraction of the toxine, the prophylactic consisting of a mixture of the products of both of these procedures carefully heated at such a temperature as to change the larger portion of the toxine into toxoid. In his experiments Macfadyen obtained a toxine from the typhoid bacillus which would kill guinea pigs in intraperitoneal doses of two-tenths cubic centimeter, and which in monkeys gave rise to an immune serum. One-tenth cubic centimeter of this serum protected guinea pigs against a fatal dose of the toxine. My own experiments show that with the modified and extracted toxine no local reaction, similar to that produced by the living or the killed cholera organisms, is obtained, even when sufficient amounts of the former are injected to cause death. The problem, therefore, which confronts us, is the extraction of the toxine in larger quantities. From a few preliminary experiments, already performed with crude apparatus, it would seem that, when the appliances and the methods recommended by

Macfadyen are employed its isolation should be more successful. My experiments throw little light on the nature of the structure of this intracellular toxine. That the haptophore group is identical in structure with that of the soluble toxines of diphtheria and tetanus bacilli would appear doubtful.

The effect of boiling upon the prophylactic.—In Table XII there is recorded a series of experiments [1] showing the effect of boiling upon the toxic action of the prophylactic when injected into rabbits. From these experiments it is seen that the toxic action was destroyed by a temperature of 100° C., since, after the prophylactic had been thoroughly boiled, neither an intravenous injection of 1 cubic centimeter nor one of 2 cubic centimeters caused the death of the rabbits inoculated with it; while control animals receiving 1 cubic centimeter of the unboiled prophylactic always died. Control animals receiving 2 cubic centimeters of peptone solution of the same specific gravity as the prophylactic were unaffected. Animals which received the boiled prophylactic were apparently but little disturbed by the inoculation, and their blood showed practically no agglutinative action with the virulent strain.

Effect produced upon toxic action of prophylactic by its preservation with chemicals.—After several weeks' preservation of the prophylactic in chloretone at room temperature, it was found that a loss of toxic power had resulted, and that evidently there had been a further change of the toxine into toxoid. From the experiments recorded in Table XIII, it is apparent that after preservation of the prophylactic for three months in chloretone neither 1 cubic centimeter nor even 2 cubic centimeters of it, when injected intravenously into rabbits, caused the death of these animals; though the injection of the latter amount produced illness with a rise of temperature of about two degrees. On comparing these results with those obtained by the use of the fresh prophylactic, we see from animal No. 439 in Table IX that formerly ½ cubic centimeter of this prophylactic brought about death, and that 1 or 2 cubic centimeters always produced this effect. (See animals Nos 438, 467, etc.) However, the agglutinable substance of the prophylactic preserved for three months in chloretone remained apparently unchanged in both its groups; since relatively the same amounts of agglu-

[1] In connection with the following experiments I wish to express my thanks to Mr. Charles B. Hare, Assistant Bacteriologist in this Laboratory, for much aid.

TABLE No. XII.—*Showing the effect of boiling upon the toxic action of the prophylactic.*

Rabbit No.	Inoculated intravenously with—	Result to animal
466	1 c. c. boiled virulent prophylactic VI (1 c. c. = 8 oesen), plus 1 c. c. NaCl solution.	Apparently unaffected by injection; killed by bleeding after one week; agglutination experiments (with virulent organism only, 1 to 600, 1 to 800, 1 to 1,000, 1 to 1,200, all negative.
467	1 c. c. of unboiled virulent prophylactic VI	Death within 24 hours.
469	1 c. c. of unboiled virulent prophylactic VI, plus 1 c. c. NaCl solution.	Do.
470	1 c. c. boiled virulent prophylactic VI, plus 1 c. c. NaCl solution	Apparently unaffected by injection; alive after one week.
471	2 c. c. peptone solution of same specific gravity as virulent prophylactic VI (for control).	Do.
473	1 c. c. virulent prophylactic VI, plus 1 c. c. NaCl solution	Death within 24 hours.
495	2 c. c. boiled virulent prophylactic VI	Apparently but little affected by injection; alive after one week.
505	2 c. c. virulent prophylactic VII (1 c. c. = 8 oesen)	Death within 24 hours.
506	2 c. c. boiled virulent prophylactic VII	Apparently but little affected by injection; alive after one week.
508	2 c. c. boiled virulent prophylactic VII	But little affected by inoculation; killed by bleeding after one week; agglutination experiments (with virulent organism only) 1 to 200, 1 to 300, 1 to 400, 1 to 500 1 to 600, all negative.

TABLE No. XIII.

Showing gradual loss of toxic action of prophylactic after three months preservation with chloretone.

Rabbit No.	Inoculated with	Result to animal.	Agglutination Experiments (with virulent organism only.) 1-100	1-200	1-300	1-400	1-500	1-600	1-700	1-800	1-1000	1-1050	1-1150	1-1200
160	1 c.c. Virulent Prophylactic VI, in chloretone (1 c.c.=8 oese.)	Apparently unaffected by injection. Killed by bleeding, one week after inoculation.						N		N	N			N
161	1 c.c. Virulent Prophylactic VI, in chloretone + 1 c.c. normal Na. Cl. solution.	1-½° of fever following injection. Killed by bleeding, one week after inoculation.								W	W			N
462	2 c.c. Virulent Prophylactic VI, in chloretone.	About 2° of fever following injection. Killed by bleeding, one week after inoculation.							W	W	N			
509	2 c.c. Virulent Prophylactic VI, in chloretone.	Sharp temp. Reaction following injection. Killed by bleeding, one week after inoculation.								W	W	N	N	N

Result to be compared with animal No. 439, Table No. IX inoculated with ½ c.c. virulent prophylactic VI, preserved for a few days only in chloretone.

tinine were produced in the sera of the rabbits inoculated with the old prophylactic as were formed in those of the animals inoculated with the fresh one, values as high as 1 milligram being obtained. (See animals Nos. 461 and 509, Table XIII.) The bactericidal values of the sera of the animals comprising Table XIII were not investigated. There was no reason to suppose that any loss in bactericidal power would be found in these sera, since it is well known that the substances giving rise to the bacteriolysines are not so unstable as those which produce the agglutinines.

In regard to the preservation of the prophylactic with 0.5 per cent carbolic acid for a long period of time, practically the same effect as that produced by chloretone has been observed, namely, that there is a weakening of the toxic action, so that, where formerly 1 and 2 cubic centimeters of the freshly prepared prophylactic produced death in a rabbit, after a long preservation in 0.5 per cent carbolic acid, 3 cubic centimeters were required to bring about such a result. (See animals Nos. 421, 422, and 434, Table IX.) However, the substances giving rise to the agglutinines and the bacteriolysines are apparently not unfavorably affected by this process. (See animal No. 423, Table VII.)

As already stated, each successive heating of the prophylactic at 60° C. or over, alike unfavorably affects the toxic as well as the agglutinable substances. When the prophylactic is boiled these are apparently destroyed. However, by a careful heating at 60° C. for fifteen minutes the toxic action apparently does not disappear entirely.

On account of the unfavorable effects of the substances mentioned above it has usually been our practice so to handle the prophylactic in its manufacture that it is received from the filter into the sterile tubes, thus making unnecessary any further sterilization either by heat or by the addition of chemicals. With the prophylactic preserved in a sterile manner and kept at the temperature of the ice box, I have obtained very good results five months after its preparation. Of course, the toxic action becomes weaker after a short period of time, and this process gradually increases, owing to the still further change of toxine into toxoid.

HUMAN INOCULATIONS.

After studying the effects of the prophylactic upon animals, it was also desirable to ascertain its action upon human beings. With

this end in view, a number of individuals have been inoculated from time to time with varying amounts (1 to 5 cubic centimeters) of the virulent prophylactic. The inoculations have been made deep into the muscles of the arm. In these cases the local reaction was never very marked. There was usually soreness on pressure in the region of the inoculation, lasting for about twenty-four hours, and occasionally a slight reddening of the overlying skin was observed. None of the patients have complained of much pain. No suppuration has ever been observed, and in fact it may be said that the local reaction is very slight. Following the inoculation there was generally a rise of temperature of from 1° to 3° (Fahrenheit), which subsided in from twenty-four to forty-eight hours. Headache, lasting for a few hours, was occasionally complained of. Unfortunately for a further trial of the method, there has not been sufficient cholera present in the city or in the provinces during the past nine months to warrant the introduction of a general inoculation of the people against this disease; nor has there been any opportunity to observe the immunity of the inoculated from an entirely practical standpoint. The fact, therefore, that no cases of cholera have occurred among those receiving the prophylactic shows nothing in regard to the value of the method, since it is doubtful to what extent they have been exposed to the disease.[1] However, it has been demonstrated that the blood sera of the inoculated individuals, both white and native, acquire protective substances. The results of a study of a number of these cases may be seen in Table XIV; before the injection the serum of none of them showed any agglutination of the virulent organism in dilutions of 1 to 20 (50 milligrams), or any bactericidal action in dilutions of 1 to 50 (20 milligrams). The blood was drawn from one of the veins of the arm one week after the inoculation, and after the separation of the serum the value of the latter was determined. From these experiments it appears that 3 or 4 cubic centimeters of the prophylactic furnished the best sera, namely, those having an agglutinative value against the virulent strain of from 4 to 2.5 milligrams and a bactericidal one of from 0.33 to 0.25 milligram. These sera are much more potent than those obtained in human beings by Kolle from the subcutaneous injection

[1] Attempts at subsequent infection of the inoculated by feeding living cultures of cholera spirilla have shown themselves so unsatisfactory in the past that for this reason and other obvious ones they have not been resorted to.

TABLE No. XIV.

Human Inoculations

(For explanation see Table No. 1.)

Case No.	Inoculated Subcutaneously with	Temperature reaction	Agglutination Experiments. with virulent organism only. Dilution of Serum. 1-10 1-20 1-40 1-80 1-100 1-150 1-200 1-250 1-300 1-350 1-400 1-500	Controls No. of Solving Serum	Bactericidal Reactions. (Pfeiffer's Phenomenon.) Dilution of Serum. 1-100 1-500 1-600 1-1000 2-000 3-000 4-000 5-000 6-000	Control Animals Without Serum.
1	3 C.C. Virulent Prophylactic V. 1 c.c. = 8 oese.	101.7° F.	W N	N N	A A A A A N/D	2 control animals; reactions neg.; all dead in 24 hours.
2	3 C.C. Virulent Prophylactic V. 1 C.C. = 8 oese.	101. 4° F.	W N N	N N	A A A A N/D	1 control animal; reaction neg.; dead in 24 hours.
3	2 C.C. Virulent Prophylactic V. 1 C.C. = 8 oese.	100.6° F	W N	N N	A A A A N/D	1 control animal, reaction neg.; dead in 24 hours.
4	2 C.C. Virulent Prophylactic V 1 C.C. = 8 oese.	101 1° F	W W N	N N	A A A A A N/D	1 control animal; reaction neg., dead in 24 hours.
5	2 C.C. Virulent Prophylactic V. 1 C.C. = 8 oese.	101 4° F	W N	N N	A A A A N D	1 control animal, reaction neg., dead in 24 hours.
6	2 C.C. Virulent Prophylactic V. 1 C.C. = 8 oese.	100 7° F	N	N N	A A A N/D	1 control animal, reaction neg.; dead in 24 hours.
7	1 C.C. Virulent Prophylactic V. 1 C.C. = 8 oese.	100 4° F.	W N	N N	A A N D	1 control animal; reaction neg.; dead in 24 hours.
8	1 C.C. Virulent Prophylactic V 1 C.C. = 8 oese	101 2° F.	W W N	N N	A A A A	1 control animal, reaction neg., dead in 24 hours.
9	1 C.C. Virulent Prophylactic V. 1 C.C. = 8 oese	101 4° F.	W N	N_0 N	A A N/D	1 control animal, reaction neg., dead in 24 hours.
10	4 C.C. Virulent Prophylactic V 1 C.C. = 8 oese.	102 2° F.	W W N	N N	A A A A N/D	2 control animals, reactions neg., all dead in 24 hours.

of either the living or the killed cholera spirilla. Kolle's best sera showed a bactericidal value of from 3 to 1.5 milligrams. They also show a higher value than is usually seen in the sera of human beings who have recovered from an attack of Asiatic cholera, which, according to the investigations of R. Pfeiffer and of Kolle, may be 10 milligrams. Therefore, we might presume that a good active immunity had been acquired against the disease by the use of this prophylactic. The antitoxic value of the sera has already been discussed. In case Number 1, two cubic centimeters of the serum protected rabbits against four times the intravenous dose, fatal for these animals.

We have seen that by the subcutaneous injection of the cholera prophylactic an excellent cholera immune serum can be obtained in human beings. However, the question naturally arises, whether these individuals are protected against intestinal infection with the cholera spirillum. In other words, are they really immune to the disease Asiatic cholera? Experiments upon animals can not satisfactorily answer this query. The earlier investigations of Brieger, Kitasato, Wassermann, Haffkine, and others upon the point at issue, namely, whether animals could be rendered immune against intestinal infection with Asiatic cholera, spoke in the affirmative. However, the more recent work of Pfeiffer, Wassermann, and Sobernheim demonstrated that immunity in animals against such infection was not certainly to be obtained by the ordinary methods of immunization then in vogue. Since animals are not naturally susceptible to intestinal infection, and since it is only through artificial means that such may be produced in them, evidently the answer to our question can be given only by a practical observation of the human beings inoculated with the prophylactic during a severe and general epidemic of the disease. For this reason it was hoped that a more extensive practical demonstration of the value of the prophylactic could be given before an extended publication of the work was made.[1]

However, since the present report has been delayed nearly nine months, and as it appears that there will be no greater opportunity in the near future for a more practical test of the prophylactic in these Islands than has already been experienced, it is thought inadvisable to defer for a longer period the publication of the

[1] The results of the experimental work were presented to the Manila Medical Society at the meeting of September 7, 1903.

experimental work. Moreover, it would appear, from the numerous statistics of Haffkine in India, and the more recent work of Murata in Japan, that simply by the injection of a small amount of the killed organisms a certain degree of immunity against the natural mode of infection is acquired. Therefore, judging from what has already been said, it is probable that by the use of our prophylactic, human beings may acquire a good active immunity against the disease.

CONCLUSIONS.

(1) By the autolytic digestion of carefully killed cholera spirilla in an aqueous fluid the receptors become separated from the bacterial cells and may be filtered off in solution.

(2) The injection of these free receptors into both man and animals furnishes a means of producing high bactericidal and agglutinative blood sera. The antitoxic value of these sera is, however, moderate.

(3) The subcutaneous injection into man of such free receptors is a process which is not only free from any danger but one which produces practically no local disturbance and only a slight general reaction.

(4) Hence the method is a practicable one for producing a cholera immune serum in man.

(5) It is highly desirable that this cholera prophylactic be given a thorough, practical test.

(6) It would appear hopeful that by the application to the pest bacillus of a slight modification of this method a more satisfactory prophylactic against bubonic plague could be obtained.

Experiments with this end in view have already been commenced in the Biological Laboratory.

The consideration of the comparative results in immunity obtained with the inoculation of the virulent and the avirulent living organisms and with the prophylactics of different virulence will be considered in another article.

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